VOLUME 33 SEPTEMBER 1955 NUMBER 5

Canadian Journal of Biochemistry and Physiology

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

CANADIAN JOURNAL OF BIOCHEMISTRY AND PHYSIOLOGY

(Formerly Canadian Journal of Medical Sciences)

Under the authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research, the National Research Council issues THE CANADIAN JOURNAL OF BIOCHEMISTRY AND PHYSIOLOGY and six other journals devoted to the publication of the results of original scientific research. Matters of general policy concerning these journals are the responsibility of a joint Editorial Board consisting of: members representing the National Research Council of Canada; the Editors of the Journals; and members representing the Royal Society of Canada and four other scientific societies.

The Canadian Physiological Society has chosen the Canadian Journal of Biochemistry and Physiology as its official journal for the publication of scientific papers.

Manuscripts for publication should be submitted to Dr. Léo Marion, Editor-in-Chief, Canadian Journal of Biochemistry and Physiology, National Research Council, Ottawa 2, Canada.

(For instructions on preparation of copy, see **Notes to Contributors** (inside back cover).)

Proof, correspondence concerning proof, and orders for reprints should be sent to the Manager, Editorial Office (Research Journals), Division of Administration, National Research Council, Ottawa 2, Canada.

Subscriptions, renewals, requests for single or back numbers, and all remittances should be sent to Division of Administration, National Research Council, Ottawa 2, Canada. Remittances should be made payable to the Receiver General of Canada, credit National Research Council.

The journals published, frequency of publication, and prices are:

Canadian Journal of Biochemistry and Physiology	Bimonthly	$3.00 a year
Canadian Journal of Botany	Bimonthly	$4.00
Canadian Journal of Chemistry	Monthly	$5.00
Canadian Journal of Microbiology*	Bimonthly	$3.00
Canadian Journal of Physics	Monthly	$4.00
Canadian Journal of Technology	Bimonthly	$3.00
Canadian Journal of Zoology	Bimonthly	$3.00

The price of single numbers of all journals is 75 cents.

** Volume 1 will combine three numbers published in 1954 with six published in 1955 and will be available at the regular annual subscription rate of $3.00.*

Canadian Journal of Biochemistry and Physiology

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOLUME 33 SEPTEMBER 1955 NUMBER 5

THE OXIDATION OF TRYPTAMINE BY RAT LIVER AND OTHER TISSUE SUSPENSIONS[1]

BY T. L. SOURKES, EDITH TOWNSEND, AND GUDRUN NAN HANSEN

Abstract

Tryptamine solutions were incubated with crude suspensions of rat and guinea pig tissues in the Barcroft–Warburg apparatus. Oxygen consumption was measured. Solutions incubated for various periods of time were deproteinized with perchloric acid, and the supernatant fraction was examined in the Beckman DU spectrophotometer. Consistent and marked changes were found in the ultraviolet absorption spectrum of the tryptamine solutions. The quantitative changes have been utilized for the measurement of monoamine oxidase activity by the "substrate disappearance" method. The significance of the qualitative changes in absorption spectrum is discussed. Similar experiments with 5-hydroxytryptamine are presented. This substrate is oxidized by rat liver at about the same rate as tryptamine.

Introduction

The conventional methods for measuring monoamine oxidase (MO) activity of tissues depend upon the determination of oxygen uptake or of ammonia release in the presence of a suitable amine substrate. Of these the former is less reliable since side reactions occur which may increase the amount of oxygen consumed (for example oxidation of the aldehyde product of MO action) or decrease the apparent uptake (for example catalatic breakdown of the hydrogen peroxide formed). Disappearance of a substrate like tyramine or adrenaline has been employed (9), but the pharmacological assay is inconvenient for purposes of following the course of a reaction and, furthermore, bears its own experimental error in addition to that of the enzymatic assay. Investigation of new, independent techniques for use with this enzyme is desirable, especially in studies of its mechanism of action or in its purification.

In the course of a study of the ultraviolet spectrophotometric changes encountered during enzymatic oxidation of aromatic amines it has been found that the absorption spectrum of tryptamine solution, in the presence of a suitable source of MO, undergoes certain characteristic changes. One of

[1] *Manuscript received April 7, 1955.*

Contribution from The Allan Memorial Institute of Psychiatry, McGill University, Montreal, Quebec. This work was aided by Federal–Provincial Mental Health Grants, Nos. 604-5-12 and -15 combined, to Dr. R. A. Cleghorn. A preliminary report of some of the material in this paper was given at the Toronto meeting of the Canadian Physiological Society, October 22, 1954.

these changes is a decrease in the absorbancy of the tryptamine solutions as a function of time. In the following report this property has been explored for its possible utility in the study of MO. Comparative data are presented employing the manometric method (oxygen utilization).

Experimental

Various tissues of rat and guinea pig were used as sources of MO. Rat liver was found most convenient for our initial work, and was prepared as suspensions of the fresh or frozen tissue or by dialysis of such preparations. The tissue was suspended in water or 0.02 molar (*M*) phosphate buffer, pH 7.4, using a glass or Teflon homogenizing pestle, and was added to chilled Warburg flasks containing additional buffer in the main well; substrate (7–40 μmoles (μM.)) in one side arm; and 0.2 ml. of 60% perchloric acid in the other. The flasks had KOH papers in the center well and were gassed with oxygen. At equilibration the initial reading was taken and the substrate was then tipped in from the side arm. In control flasks water was substituted for tryptamine (tissue blanks). At the end of the desired incubation period the perchloric acid was tipped to stop the reaction and to precipitate proteins. The deproteinized reaction mixtures were suitably diluted to give in the 'zero time' flask about 10^{-4} *M* tryptamine, and these solutions were then examined in the Beckman DU spectrophotometer. By stopping the reaction in pairs of flasks (one containing substrate, the other not) at various time intervals, a continuous record of oxygen uptake and a discontinuous record of changes in the absorption spectrum of the substrate solutions was obtained. The absorbancy of the solutions at 279 mμ or at 287 mμ permitted calculation of the amount of tryptamine remaining in the solution. A reading of 0.570 at 279 mμ or of 0.474 at 287 mμ corresponds to a concentration of 0.1 μM. of tryptamine per ml. For serotonin a reading of 0.605 at 276 mμ was taken to indicate a concentration of 0.1 μM. per ml.

Dry weights of tissue suspensions were determined by heating 1 ml. aliquots to constant weight at 105°. These data were used to calculate $-Q$ values ($-Q$ = μl. of oxygen consumed or of substrate disappearing per mgm. dry weight per hour). Standard manometric techniques (13) were used in the Warburg determinations of MO activity. All experiments were carried out at 38°; the gas phase was oxygen except where otherwise specified.

Tryptamine hydrochloride was purchased from the Eastman Kodak Co. Dr. K. E. Hamlin, Jr., of Abbott Laboratories, Chicago, kindly supplied the serotonin creatinine sulphate used in this work.

Results

1. *Changes in the Ultraviolet Absorption Spectrum of Tryptamine During Enzymatic Oxidation*

In plotting absorption spectra the absorbancy of the deproteinized and diluted tryptamine solutions was corrected by subtraction of the absorbancy

of the similar treated paired control ('tissue blank') flask. Alternatively, the instrument was set to zero absorbancy with the latter and the tryptamine sample was read against this.

Oxidation of tryptamine was accompanied by a progressive decrease in optical density (O.D.) at the two absorption maxima, 279 mμ and 287 mμ. A new peak gradually appeared at 272 mμ (Fig. 1). These changes were also noted when an aldehyde reagent such as semicarbazide was present. A number of experiments were performed using 5-hydroxytryptamine (serotonin). With this compound the absorbancies at 276 mμ and 322 mμ[3] both decrease with time of incubation. The peak shifts to 274 mμ. The inflection at 292–3 mμ flattens to form a shoulder.

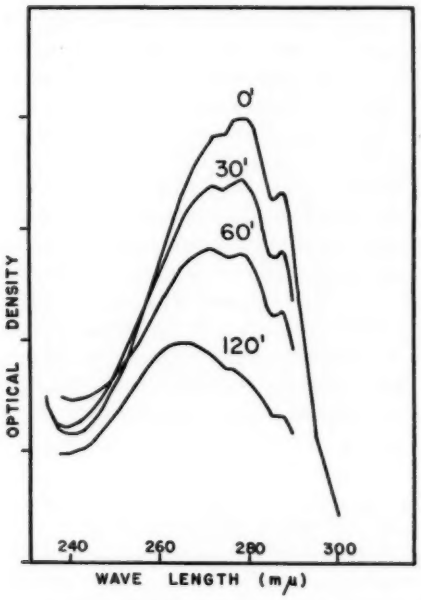


FIG. 1. Absorption spectrum of tryptamine solutions incubated for various periods of time with fresh rat liver suspension, pH 7.4, 38°. In this particular experiment the optical density of the zero time reaction mixture, diluted suitably for spectrophotometry, was 0.600.

Thus, two types of change have been noted in the absorption spectrum of the indolylethylamines: (1) qualitative changes, characterized by the shifting of peaks; and (2) decrease in the absorbancy at the characteristic peaks. It may be well to discuss these in their order.

The qualitative changes undoubtedly represent a complex of processes, including side reactions. The latter are indicated by the pigment formation which occurs with both tryptamine (12) and 5-hydroxytryptamine (3).

[3] *The latter peak is evident at pH 12.*

Little is known about the mechanism of this pigment formation. The amounts of pigment are decreased in the presence of aldehyde reagents (4). Pugh (12) has suggested that oxidations of the indole ring are involved. Blashko and Philpot (5) consider that serotonin may undergo oxidation to a quinone–imine structure (phenolic group and indolic nitrogen). If side reactions occur to a significant extent then changes in the absorption spectrum of these amines in the course of oxidation are to be anticipated. The alteration in the serotonin spectrum at 292–3 mμ may be indicative of such a reaction.

Besides the oxidation of indolic substance(s) there is also the metabolism of ultraviolet-absorbing material in the liver itself to consider. The liver preparations used show an absorption peak at or near 260 mμ; during the incubation in the Warburg flasks the absorbancy at this wavelength decreases with time. This process occurs anaerobically, although it is slightly accelerated under oxygen. If the absorbancy in the region around 260 mμ falls less rapidly than in the 275–290 mμ region where the specific peaks of tryptamine occur, then the ultimate appearance of a new peak, to which the endogenous ultraviolet-absorbing material contributes heavily, can be expected. This is supported by the data of Table I and Fig. 1. Moreover, in control experiments with varying amounts of tryptamine the ratio of the absorbancies at 287 and 279 mμ has been found to be 0.83. In most of the incubation experiments this ratio has been maintained, but in a few the ratio increases to above 1.0. This differential change in absorbancies at the two wavelengths suggests that in these particular experiments changes other than decrease in tryptamine (i.e. structural changes) are going on.

The decrease in absorbancy of the incubated tryptamine solutions at the characteristic peak wavelengths suggests that indolic material is being removed

TABLE I

OXYGEN CONSUMPTION AND SPECTROPHOTOMETRIC CHANGES OF TRYPTAMINE SOLUTIONS UNDERGOING ENZYMATIC OXIDATION

Expt. No.	Enzyme and additions		Incubation time (min.) 0	30	60	120
1	Fresh rat liver	μM.O_2	0		3.0	5.0
		O.D. ratio[a]	0.94		0.97	1.01
2	Rat liver, dialyzed 3 hr.[b]	μM.O_2	0	4.3	6.8	
		O.D. ratio	0.96	0.99	1.05	
3	Same as No. 2 plus hydroxylamine[c]	μM.O_2	0	3.4	6.8	13.3
		O.D. ratio	0.92	0.96	1.00	1.04
4	Fresh rat liver	μM.O_2	0	1.3		3.7
		O.D. ratio	0.96	1.00		1.11
5	Fresh guinea pig liver	μM.O_2	0	6.4		
		O.D. ratio	0.95	1.00		

[a] *O.D. at 272 mμ/O.D. at 279 mμ.*
[b] *Against 0.02 M phosphate buffer, pH 7.4.*
[c] *0.001 molar, final concentration.*

either before or during the precipitation of the proteins by perchloric acid. Control experiments with complete reaction mixtures, including liver suspension, show that added tryptamine is 95–100% recovered after the precipitation. The observed decrease in absorbancy of incubated tissue–tryptamine mixtures may simply be a removal (partial or complete) of the oxidative products with the protein precipitate. When the deproteinized solutions are chromatographed on paper using *n*–butanol/acetic acid/water (4 : 1 : 1) as solvent, the area of the spot in the tryptamine position decreases regularly with time. This provides further evidence that the decrease in O.D. at 279 or 287 mμ is a measure not merely of the disappearance of "indolic substances, but more specifically of tryptamine.

2. *Spectrophotometric Determination of MO Activity*

The consistency with which the changes were noted in the various experiments prompted us to evaluate the spectrophotometric determination of decrease in indolic absorption as a new method of measuring MO activity. It is clear from the discussion in Section 1 that two factors may militate against obtaining a true picture of tryptamine oxidation by the spectrophotometric method. These are: (1) the unpredictable contribution by the endogenous ultraviolet-absorbing material to absorbance at 279 or at 287 mμ; and (2) incomplete removal of the products of oxidation. These sources of error can be considered in terms of several theoretical possibilities:

I. If the metabolism of the endogenous ultraviolet-absorbing material is unaffected by the presence of tryptamine or its oxidation products then the difference between the absorbancies of the 'tryptamine flasks' (A_a = absorbancy contributed by tryptamine plus endogenous liver material) and the 'tissue blanks' (A_b), respectively, gives the correct value of the absorbancy due to tryptamine remaining after oxidation for a given period of time.

II. If the metabolism of this material is suppressed in the presence of tryptamine undergoing oxidation then A_b is lower than the actual contribution by "endogenous" to A_a. Hence, $A_a - A_b$ overestimates the amount of tryptamine remaining, and underestimates the rate of oxidation. This result also occurs if the indolic oxidation product of tryptamine is not completely removed.

III. If the endogenous material decreases faster in the presence of tryptamine, for example by a coupled reaction with the peroxide formed, it can be shown that the rate of tryptamine oxidation will then be overestimated.

In practice, the error stemming from variations in content of endogenous material can be minimized by making all the absorbancy readings at higher wavelengths, relatively distant from the peak of "endogenous" absorption. Thus at 279 mμ, and even more so at 287 mμ, A_b has decreased to small values relative to A_a.

As a further check on the error of the method the ratio of oxygen consumption to tryptamine "disappearance" can be used. From consideration of the stoichiometry of the MO reaction the theoretical ratio is 1.0; this is reduced

toward the value of 0.5 if catalase is present. If the aldehyde is further metabolized the respective ratios would be 2.0 and 1.0. That is, the oxygen/substrate ratio can be expected to vary between the outside limits of 0.5 and 2.0 in crude tissue preparations. From 16 experiments with aqueous suspensions of liver in which tryptamine disappearance has been measured by the spectrophotometric method the ratio found has averaged 0.74, with a standard error of 0.07.

3. *MO Activity of Rat and Guinea Pig Tissues*

Some typical figures for MO activity of crude tissue suspensions are shown in Table II. The order of MO activity of the rat and guinea pig tissues studied here (liver > kidney > brain) is the same in sheep (1) and in man (2, 11). Pig kidney, however, has higher activity than pig liver (1).

TABLE II

MONOAMINE OXIDASE ACTIVITY OF TISSUES

Species	Tissue	$-Q_{O_2}$ or $-Q_T$[a]	Incubation period (min.) 15	30	60
Rat	Liver	O_2	3.9	2.5	2.7
		T	—	4.7	3.7
		O_2/T	—	0.53	0.73
	Kidney	O_2			0.7
		T			0.5
		O_2/T			1.4
Guinea pig	Liver	O_2	18.0	13.5	
		T	18.5	14.1	
		O_2/T	0.97	0.96	
	Kidney cortex	O_2		4.2	
		T		4.6	
		O_2/T		0.91	
	Cerebral cortex	O_2		1.2	
		T		1.9	
		O_2/T		0.63	

[a] *$-Q$ value for tryptamine.*

4. *Effect of Partial Pressure of Oxygen*

The data of Table III illustrate the parallelism between the manometric method and the method of "substrate disappearance" in relation to gas phase in the Warburg flasks. There is no detectable change in the ultraviolet absorption spectrum of tryptamine up to 40 min. under nitrogen.

TABLE III

EFFECT OF PARTIAL PRESSURE OF OXYGEN ON THE OXIDATION OF TRYPTAMINE BY RAT LIVER SUSPENSIONS

Gas phase	Time (min.)	$-Q_{O_2}$	$-Q_T$	Ratio
Nitrogen	40	0.0	0.0	—
Air	60	1.7	2.4	0.7
Oxygen	60	2.6	5.5	0.5

5. *Effect of Freezing and Dialysis*

Using fresh rat liver preparations the rate of disappearance of indolic material, estimated as tryptamine, tends to fall off with time somewhat faster than does the oxygen uptake (Table IV). With frozen or dialyzed liver preparations there is frequently a greater disparity in the rates, especially in the initial period (Table IV). In these cases the tissue consumes oxygen at a high rate in the first 20–30 min., and then the rate gradually falls off. On the other hand tryptamine disappearance exhibits a lag period (see expecially expts. 4, 5, 7, and 9, Table IV), followed by a more rapid and steadier rate in the succeeding period, up to two hours (see expts. 5, 6, and 8, Table IV).

TABLE IV

RELATION OF OXYGEN CONSUMPTION TO TRYPTAMINE DISAPPEARANCE IN RAT LIVER SUSPENSIONS

Expt. No.	Tissue prep'n.	μM. substrate added	μM.	Incubation period (min.) 15	20	30	45	60	90	120
1	Fresh	15	O_2[a]	1.1		1.7	3.3	4.2		
			T	—		3.5	3.8	5.5		
			Ratio	—		0.5	0.9	0.8		
2	Fresh	7	O_2			1.1	1.5		2.7	3.7
			T			1.7	2.2		3.6	4.4
			Ratio			0.6	0.7		0.8	0.8
3	Fresh	15	O_2				5.5			11.5
			S[b]				6.6			8.3
			Ratio				0.8			1.4
4	Frozen[c]	15	O_2	1.9		3.4				
			T	1.3		4.4				
			Ratio	1.5		0.8				
5	Frozen	15	O_2			1.0		2.1		4.0
			T			0.8		2.0		4.4
			Ratio			1.3		1.1		0.9
6[d]	Frozen	40	O_2	4.4		6.3	13.6			
			T	3.9		6.7	13.6			
			Ratio	1.1		0.9	1.0			
7	Frozen	10	O_2		1.8		2.9[e]			
			T		0.6		1.4			
			Ratio		3.0		2.1			
8	Dialyzed[f] 3 hr.	20	O_2			4.3		6.8		10.4
			T			2.0		4.0		8.3
			Ratio			2.2		1.7		1.3
9	Dialyzed	20	O_2		1.6			3.3		4.0
			T		0.1			1.4		5.4
			Ratio		16.0			2.4		0.7
10	Dialyzed 18 hr.	40	O_2		2.1		4.3[e]	5.0		8.6
			T		1.7		3.5	4.6		6.1
			Ratio		1.2		1.2	1.1		1.4

[a] *Extra O_2 uptake, i.e. (O_2 consumption in presence of tryptamine) minus (uptake in absence of substrate).* [b] *Serotonin.* [c] *Held at −10° C. for 24 hr.* [d] *Guinea pig liver.* [e] *40 min.* [f] *Dialyzed against 0.02 M phosphate buffer, pH 7.4.*

6. *Effect of Tryptamine on the Endogenous Respiration of Liver Suspensions*

If large amounts of liver suspension are used or if the suspensions are made up in isotonic potassium chloride (to protect the integrity of the actively respiring mitochondria) the rate of endogenous respiration may be so high that the addition of tryptamine causes no increase in the rate or even depresses it. Thus, by the manometric method MO activity can not be detected, although by spectrophotometric measurement considerable activity may be present (Table V, expt. 1). A similar type of result is obtained when certain sulphydryl compounds are added (10) to the reaction mixtures. Expts. 2 and 3, Table V, show that tryptamine in the presence of added cysteine causes a decrease in, rather than a stimulation of oxygen consumption; yet it is lost from the solution at a substantial rate. An experiment (No. 4) in which the tissue and amine are shaken under air instead of oxygen provides a further illustration. These figures (Table V) thus show that tryptamine under certain conditions interferes with the endogenous respiratory process in the crude suspension while itself undergoing oxidation.

TABLE V

APPARENT DISCREPANCIES BETWEEN MANOMETRIC AND SPECTROPHOTOMETRIC MEASUREMENTS OF MONOAMINE OXIDASE ACTIVITY

Expt. No.	Measurement made	Incubation time (min.) 15	30	50	60	90
1	μM.O_2 uptake − T[a]	7.8	13.0		22.0	31.2
	+ T	5.9	8.4		11.7	14.7
	μM.T lost	2.0	2.4		4.3	6.0
2	μM.O_2 uptake − T	3.2				
	+ T	2.5				
	μM.T lost	0.5				
3	μM.O_2 uptake − T				2.5	
	+ T				2.1	
	μM.T lost				1.7	
4	μM.O_2 uptake − T			6.4		
	+ T			2.5		
	μM.T lost			1.3		

Expt. 1: 200 mgm. wet weight of rat liver suspended in isotonic KCl.
Expts. 2 and 3: 100 mgm. wet weight of liver; 4 μM. of cysteine added.
Expt. 4: incubated under air instead of oxygen.
[a] − T = *'tissue blank'*; + T = *tryptamine added as substrate.*

7. *Comparison of Tryptamine and 5-Hydroxytryptamine as Substrates for MO*

Using a number of mammalian and invertebrate tissues Blaschko and Philpot (5) found that 5-hydroxytryptamine is oxidized by MO at a rate that is slow compared to the rate of oxidation of tryptamine or tyramine. Table VI summarizes our data obtained with suspensions of fresh rat liver, a tissue not reported by the above authors. The 5-hydroxytryptamine, employed as the creatinine sulphate salt, was oxidized somewhat faster than tryptamine as measured either by the manometric or the spectrophotometric method, in five of seven comparison experiments.

TABLE VI

RATES OF OXIDATION OF TRYPTAMINE AND 5-HYDROXYTRYPTAMINE BY RAT LIVER SUSPENSIONS

Expt. No.	Incubation period (min.)	Substrate			
		Tryptamine		5-Hydroxytryptamine	
		$-Q_{O_2}$	$-Q_T$	$-Q_{O_2}$	$-Q_{HT}$
1	30	3.0	4.5	3.7	6.6
2	20	1.3	—	2.3	—
3	20	1.8	—	3.4	—
4	20	2.2	—	1.8	—
5	20	1.6	—	3.1	—
6	60	4.4	3.1	4.3	2.7
7	60	1.7	1.6	2.3	1.7

Discussion

The method of determination of MO described in this paper gives results which agree reasonably well with those obtained by measurement of the stimulation of oxygen uptake by an amine. Under a variety of test conditions, the absolute rate of disappearance of tryptamine (or of 5-hydroxytryptamine) determined by spectrophotometry is greater than the rate of oxygen uptake. This is not always so, however, for frozen or dialyzed rat liver preparations; in these the decrease in tryptamine may lag behind the uptake of oxygen. The reason for this difference requires further study. If it is because the aldehyde product of the MO reaction is incompletely removed by the frozen or dialyzed tissues then it should be possible to detect in the fresh tissue some substance which binds this aldehyde.

The advantage of the spectrophotometric measurement of substrate disappearance is shown in experiments conducted under conditions in which the "extra oxygen consumption" due to the added amine is suppressed (Table V). Where the manometric method is unable to detect MO activity, the spectrophotometric examination of the incubated solutions indicates that tryptamine is nevertheless being removed at a substantial rate.

One possible application of the method is in the purification of the enzyme. Experiments are under way to determine whether the reaction can be carried out in Beckman cuvettes, permitting a continuous record of the oxidation. This technique may be facilitated by the discovery that liver MO, associated with the mitochondria (6, 8), can be brought into solution by the use of certain detergents (7).

References

1. BHAGVAT, K., BLASCHKO, H., and RICHTER, D. Biochem. J. (London), 33 : 1338. 1939.
2. BIRKHAUSER, H. Helv. Chim. Acta, 23 : 1071. 1940.
3. BLASCHKO, H. Biochem. J. (London), 52 : x. 1952.
4. BLASCHKO, H. and HELLMANN, K. J. Physiol. (London), 122 : 419. 1953.

5. BLASCHKO, H. and PHILPOT, F. J. J. Physiol. (London), 122 : 403. 1953.
6. COTZIAS, G. C. and DOLE, V. P. Proc. Soc. Exptl. Biol. Med. 78 : 157. 1951.
7. COTZIAS, G. C., SERLIN, I., and GREENOUGH, J. J. Science, 120 : 144. 1954.
8. HAWKINS, J. Biochem. J. (London), 50 : 577. 1952.
9. HOLTZ, P., HEISE, R., and SPREYER, W. Arch. exptl. Pathol. Pharmakol. 188 : 580. 1938.
10. LAGNADO, J. and SOURKES, T. L. Unpublished data.
11. LANGEMANN, H. Helv. Physiol. et Pharmacol. Acta, 2 : 367. 1944.
12. PUGH, C. E. M. and QUASTEL, J. H. Biochem. J. (London), 31 : 2306. 1937.
13. UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F. Manometric techniques and related methods for the study of tissue metabolism. Burgess Publishing Company, Minneapolis. 1949.

EFFECTS OF α-METHYL-DL-TRYPTOPHAN ON THE OXIDATION OF TRYPTOPHAN[1]

By T. L. Sourkes and Edith Townsend

Abstract

α-Methyl-DL-tryptophan (AMT) inhibits the oxidation of DL-tryptophan by isotonic suspensions of rat liver, and the oxidation of L-tryptophan, L-tyrosine, DL-phenylalanine, and DL-methionine by cobra venom. The oxidation of L-tryptophan is accompanied by the appearance of a yellow color in the reaction mixtures. AMT also inhibits the formation of kynurenine, detected by its specific absorption at 360 mμ and by diazotization, from L-tryptophan. It protects the kynurenine-forming enzyme system against the inactivation which occurs in the absence of substrate. When injected into the rat, AMT, like tryptophan, causes an adaptive increase in the tryptophan peroxidase–oxidase complex. AMT is not oxidized by the liver and venom preparations used.

Introduction

A number of α-methyl-α-amino acids have been studied in recent years for their effect on metabolic reactions. The subject has been reviewed by Umbreit (18). Recently, some new branched-chain amino acids have been found to occur naturally, a fact which points up the significance of studies with the α-methyl-α-amino acids. Done and Fowden (3) isolated γ-methylene glutamic acid from peanuts. Fink *et al.* (5, 6) have found α-amino-isobutyric acid (α-methyl-β-alanine) in urine following the administration of thymine, desoxyribonucleic acid, or dihydrothymine to rats. 3-Methylhistidine is a new amino acid identified in human urine (17). α-Methylserine is a constituent of the antibiotic Amicetin (7). Because of the possible utility of substituted amino acids in elucidating mechanisms of enzymatic action a study was made of the effect on tryptophan oxidation of the branched-chain homologue, α-methyl-DL-tryptophan (AMT). This compound has already been shown to be inert toward tryptophanase (9).

Experimental

Standard manometric techniques (19) were used in this work for measuring oxygen consumption. Rat tissues were suspended in isotonic KCl using a Teflon pestle and glass homogenizing tube and were added in suspension to chilled Warburg flasks containing phosphate buffer and AMT (or water in control flasks). Substrates were held in the side arm of the flask until gassing and equilibration of the flasks to 38° had been accomplished, after which they were tipped into the main well. For the tryptophan-to-kynurenine system (consisting of a peroxidase–oxidase and a formylase (cf. ref. 10)) the suspensions of rat liver were centrifuged at 2° for 15–20 min. at 20,000 × *g*, the supernatant solution being used according to the prescription of Knox and Mehler (10). Kynurenine was determined by its absorption at 360 mμ (10)

[1] *Manuscript received April 13, 1955.*
Contribution from The Allan Memorial Institute of Psychiatry, McGill University, Montreal, Quebec. This work was aided by a grant to Dr. R. A. Cleghorn from the Federal–Provincial Mental Health Research Fund.

or by a modification of the Bratton-Marshall technique for sulphonamides (8), employing a calibration curve made up with kynurenine and reading at 560 mμ.

The AMT was synthesized in the Research and Development Laboratories of Merck and Co., Inc., Rahway, N.J., by Mr. William J. Leanza and Dr. Karl Pfister, III.

Results

1. Effect of AMT on Endogenous Respiration of Liver Preparations

Endogenous respiration of rat liver suspensions was measured as oxygen consumption in the absence of added substrate. The reaction mixture used for this determination contained 100 mgm. wet weight of tissue, 0.02 molar (*M*) phosphate buffer, pH 7.4, 0.07 *M* KCl, and 0.01 *M* AMT (final volume, 2.0 ml.). The gas phase was air. Oxygen consumption under these conditions went on at a steady rate for 30–45 min.; the uptake was lower in the presence of AMT than in its absence. In nine experiments, the decreases below control values were 30, 51, 17, 23, 25, 42, 41, 4, and 15% respectively. Using the crude enzyme of Knox and Mehler, i.e. the supernatant fraction of centrifuged liver suspensions (see 'Experimental'), no stimulation of oxygen uptake was seen. This was frequently the case even when tryptophan was added to such preparations (cf. 10, and see below). In all cases where the crude kynurenine-forming system was employed with AMT the neutral deproteinized filtrates of the reaction mixtures were examined for kynurenine-like material. In a few cases the absorption at 360 mμ increased during one hour's incubation, particularly with concentrations of AMT of about 0.002–0.004 molar (*M*). However, numerous attempts to detect the appearance of diazotizable material following incubation of AMT with the liver fraction gave negative results. It has been tentatively concluded, therefore, that AMT does not undergo oxidation by rat liver to α-methylkynurenine.

2. D-*Amino Acid Oxidase of Rat Liver*

AMT inhibited the oxidation of DL-tryptophan by isotonic rat liver suspensions (Table I). Although L-tryptophan is oxidized by rat liver,

TABLE I

OXIDATION OF DL-TRYPTOPHAN BY ISOTONIC RAT LIVER SUSPENSIONS

Results expressed as Q_{O_2} (μl. O_2 consumed per mgm. dry wt., per hr.), based on 30 min. uptake; corrected for endogenous respiration. Final concentration of AMT, 0.01 *M*

	AMT	
Expt. No.	Absent	Present
1	1.5	0.1
2	3.2	1.8
3	2.1	1.6

considerably more concentrated extracts than those used here are required to demonstrate it. Hence this oxidation is attributed primarily to the D-amino acid oxidase of rat liver.

3. Cobra Venom Experiments

The oxidation of L-tryptophan by the L-amino acid oxidase of cobra venom was inhibited markedly by AMT. The data for this and three other amino acids are shown in Table II. It was found that the contents of the flasks containing tryptophan developed a yellow color in the course of the oxidation. This does not appear to have been observed by others working with snake venoms (1, 21). Since the uptake of oxygen with tryptophan as substrate is greater than the theoretical, as found by Bender and Krebs (1) and confirmed in our work[2], there are possibilities of other oxidations than that at the α-carbon atom.

TABLE II

OXIDATION OF AMINO ACIDS BY COBRA VENOM

Results expressed as μl. O_2 absorbed in 60 min.

	AMT			
	Expt. 1*		Expt. 2	
Substrate	−	+	−	+
None	0	5	11	11
L-Tryptophan	138	88	61	49
DL-Phenylalanine	59	38	76	42
L-Tyrosine	60	44	84	54
DL-Methionine	58	52	51	28

** Reaction mixtures: 1.0 ml. of tris(hydroxymethyl)-aminomethane buffer, 0.2 M, pH 7.4; 0.4 ml. of AMT, 0.05 M; 0.4 ml. of substrate (0.1 M DL-methionine, others added as finely ground suspensions in water); water to 2.0 ml. Expt. 1, 1.7 mgm. cobra venom per flask; Expt. 2, 1.0 mgm. per flask.*

The rate of oxygen uptake in the venom experiments was constant for two hours. There was no oxidation of AMT.

4. Kynurenine Formation from L-Tryptophan

In our work with the enzyme system oxidizing tryptophan to kynurenine we have found that the amount of kynurenine formed is proportional to the concentration of L-tryptophan up to 0.0016 *M*. Higher concentrations depress kynurenine production slightly. As mentioned previously AMT is not oxidized by this system. However, it inhibits the formation of kynurenine

[2] *Total O_2 uptake at completion of oxidation of tryptophan was 8, 13, 11, and 12% respectively, greater than theoretical in four experiments.*

TABLE III

EFFECT OF AMT ON KYNURENINE FORMATION FROM L-TRYPTOPHAN

Expt. No.	L-Tryptophan (μM.)	AMT (μM.)	Kynurenine formed (μM./hr.)
1	4	0	0.83
	4	12	0.63
	4	24	0.45
2	4	0	0.38
	4	30	0.22
3	8	0	0.39
	8	5	0.40
	8	10	0.36
	8	30	0.25
4	8	0	0.56
	8	34	0.46
5	10	0	1.23
	10	5	0.37
	10	10	0.45

Reaction mixtures: 2.0 ml. containing 0.02 M phosphate buffer, pH 7.4. AMT added to liver extract 10 min. before substrate.

from L-tryptophan (Table III). Oxygen consumption by the liver extract was measured in every experiment performed; in only the occasional case did L-tryptophan stimulate oxidation as measured manometrically. Oxygen consumption in the absence of the substrate was always very high (cf. (10)).

Knox and Mehler (10) mention that the enzyme system loses activity quickly, even in 15 min. if the liver extract is allowed to stand without substrate. The data of Table IV show that AMT protects the system from inactivation. In the experiments shown AMT and L-tryptophan were placed in separate side arms of the Warburg flasks. The AMT was tipped into the main well containing the liver extract at various times before substrate was added, or at the same time. The liver extract was thus incubated at 38° for 30 min. (Expts. 1, 2, and 3) or 20 min. (Expt. 4) before L-tryptophan was mixed with it. Since inhibition is incomplete even with 34 μM. of AMT per flask (Table III), when the enzyme is allowed to deteriorate in the absence of substrate the "protecting action" of AMT becomes evident (Table IV). If AMT and substrate are added to the liver extract simultaneously only the inhibitory effect of the former is seen. Final concentration of AMT in the experiments of Table IV was 0.02 *M*.

The tryptophan peroxidase system is an adaptive one, its content in liver increasing after tryptophan is fed to the animal (11) or after addition of tryptophan to liver slices *in vitro* (4). AMT is also capable of provoking this increase, after subcutaneous injection in the rat (Table V). This increase is prominent in four hours, and is even greater than the increase with tryptophan.

TABLE IV

"PROTECTING ACTION" OF AMT ON THE TRYPTOPHAN PEROXIDASE–OXIDASE–FORMYLASE SYSTEM OF RAT LIVER

L-Tryptophan added at zero time; final concentration 0.002 *M*. Incubation period, one hour, after addition of substrate. Results expressed as μM. of kynurenine formed in one hour

Expt. No.	Time of adding AMT (min.)	AMT		Difference, effect of AMT
		Absent	Present	
1	− 30	0.13	0.46	0.33
	− 15	0.16	0.35	0.19
	0	0.57	0.47	− 0.10
2	− 30	0.10	0.16	0.06
	− 15	0.19	0.19	0.00
	0	0.35	0.21	− 0.14
3	− 30	0.10	0.22	0.12
	− 15	0.17	0.27	0.10
	0	0.42	0.24	− 0.18
4	− 20	0.16	0.32	0.16
	− 10	0.20	0.27	0.07
	0	0.23	0.16	− 0.07

TABLE V

ADAPTIVE FORMATION OF TRYPTOPHAN PEROXIDASE–OXIDASE IN RAT

Male rats, 150 ± 5 gm. administered DL-tryptophan or DL-AMT, dissolved in saline, by subcutaneous route. Experiment 1, 1.0 mM. of compound given; experiments 2 and 3, 0.5 mM. Animals sacrificed at 4th hour after injection; livers excised and prepared as described in text. Incubation period, one hour. Results in μM. kynurenine formed from 4 μM. of L-tryptophan per hour. Diazotization procedure used. Dry weight of liver preparations, 59–62 mgm. per flask

Expt. No.	Compound injected	Kynurenine formed
1	None (saline control)	0.34
	DL-Tryptophan	1.21
	DL-AMT	1.73
2	None	0.24
	DL-Tryptophan	1.28
	DL-AMT	2.83
3	None	1.34
	DL-Tryptophan	2.69
	DL-AMT	3.75

Hypaphorine was tested in the Knox–Mehler system. It did not yield a diazotizable amine.

Discussion

The substitution of the α-H atom of tryptophan by a methyl group renders the compound resistant to oxidation by rat liver preparations and by cobra venom. It is, of course, known that the D-amino acid oxidase requires an

H atom on the α-carbon of the substrate (12); the same is true of the L-amino acid oxidases (20). Although inert as a substrate, AMT can nevertheless inhibit the oxidation of D- and L-tryptophan by the corresponding amino acid oxidases.

It is of interest in connection with the results reported here that some of the α-methyl-α-amino acids may inhibit reactions of the corresponding naturally occurring amino acids involving the α-carbon linkages, but can themselves react at sites distant from this point. For example, α-methylglutamic acid inhibits glutamic decarboxylase (14), but itself can form α-methylglutamine (2, 13). α-Methyl-3,4-dihydroxyphenylalanine inhibits DOPA decarboxylase (15), but is itself rapidly oxidized by mushroom tyrosinase (16). Moreover, these homologues are inert as substrates for reactions at the α-carbon. In the present case AMT inhibits oxidation of tryptophan at the α-carbon (as well as at the indolic N—C bond), is not a substrate for the enzymes concerned, but can substitute for tryptophan in stabilizing the enzyme system and in evoking adaptive formation of the peroxidase–oxidase.

Acknowledgments

The amino acids used were a gift from Merck & Co., Ltd., Montreal. Dr. M. Spacek kindly provided a sample of DL-kynurenine sulphate, and Dr. K. Folkers a sample of hypaphorine hydrochloride. Dr. A. D'Iørio gave us a generous supply of cobra venom.

References

1. Bender, A. E. and Krebs, H. A. Biochem. J. 46 : 210. 1950.
2. Braganca, B. M., Quastel, J. H., and Schucher, R. Arch. Biochem. and Biophys. 41 : 478. 1952.
3. Done, J. and Fowden, L. Biochem. J. 51 : 451. 1952.
4. Efimochkina, E. F. Biokhimiya, 19 : 68. 1954.
5. Fink, R. M., Fink, K., and Henderson, R. B. J. Biol. Chem. 201 : 349. 1953.
6. Fink, K., Henderson, R. B., and Fink, R. M. J. Biol. Chem. 197 : 441. 1952.
7. Flynn, E. H., Hinman, J. W., Caron, E. L., Woolf, D. O. J. Am. Chem. Soc. 75 : 5867. 1953.
8. Hawk, P. B., Oser, B. L., and Summerson, W. H. Practical physiological chemistry. 12th ed. The Blakiston Co., New York. 1947. p. 603 ff.
9. Keller, D. and Umbreit, W. W. Unpublished data. Cf. Ref. 18.
10. Knox, W. E. and Mehler, A. H. J. Biol. Chem. 187 : 419. 1950.
11. Knox, W. E. and Mehler, A. H. Science, 113 : 237. 1951.
12. Krebs, H. A. *In* Sumner, J. B. and Myrbäck, K. The enzymes. Vol. II. Pt. I. Academic Press, Inc., New York. 1951. p. 505.
13. Lichtenstein, N., Ross, H. E., and Cohen, P. P. J. Biol. Chem. 201 : 117. 1953.
14. Roberts, E. J. Biol. Chem. 202 : 359. 1953.
15. Sourkes, T. L. Arch. Biochem. and Biophys. 51 : 444. 1954.
16. Sourkes, T. L. Rev. can. biol. 14 : 49. 1955.
17. Tallan, H. H., Stein, W. H., and Moore, S. J. Biol. Chem. 206 : 825. 1954.
18. Umbreit, W. W. *In* McElroy, W. and Glass, B. Amino acid metabolism, The Johns Hopkins Press, Baltimore, Md. 1955. p. 48-54.
19. Umbreit, W. W., Burris, R. H., and Stauffer, J. F. Manometric techniques and related methods for the study of tissue metabolism. Burgess Publishing Company, Minneapolis. 1945.
20. Zeller, E. A. *In* Sumner, J. B. and Myrbäck, K. The enzymes. Vol. I. Pt. 2. Academic Press, Inc., New York. 1951. p. 1003.
21. Zeller, E. A. and Maritz, A. Helv. Chim. Acta, 28 : 365. 1945.

THE INFLUENCE OF ADRENERGIC BLOCKING AGENTS ON METABOLIC EVENTS IN HEMORRHAGIC SHOCK IN THE DOG[1]

By F. Lotz[2], L. Beck[3], and J. A. F. Stevenson

Abstract

Dibenamine hydrochloride administered intravenously 30 min. after the onset of hemorrhage in anesthetized dogs, in which hemorrhagic hypotension (43–45 mm. Hg for 90 min.) of the Wiggers' type had been produced, resulted in an increased rate of survival as compared to that of untreated controls. The mean increase in plasma amino nitrogen and the percentage decrease in total arterial oxygen transport were significantly less in the Dibenamine-treated group. In animals treated with Dibenamine or Dibenzyline hydrochloride 85 min. after hemorrhage as compared to untreated controls, there were no significant differences in survival rate, plasma amino nitrogen, lactate, pyruvate, lactate/pyruvate ratio, bicarbonate content, or blood pH. The arterial oxygen transport, however, was significantly higher after than before treatment and also in the postinfusion period was higher in the two treated groups than in the untreated group. In these two late-treated groups, however, the plasma amino nitrogen increase was significantly greater in the fatalities than in the survivors during both the hypotensive and postinfusion periods. In the untreated group, differences between survivors and fatalities appeared only after the reinfusion of the withdrawn blood.

Introduction

The abnormalities of carbohydrate and protein metabolism observed in hemorrhagic shock are largely attributed to the consequences of protracted hypoxia. A constant finding in shock is the progressive rise in plasma amino nitrogen which Engel (9) and Kline (12) have shown is an accurate index of the severity of shock and reciprocally, of the possibility of survival. Seligman *et al.* (17), however, did not find that the ability of shocked animals to clear the blood of injected amino acids bore any significant relationship to the effectiveness of transfusion or to eventual death or survival.

Less significant but nonetheless dramatic changes in carbohydrate metabolism, as evidenced by increases in lactic acid, pyruvic acid, and L/P ratio, have been extensively investigated in hemorrhagic shock (3, 13, 16, 18). Whereas it is generally agreed that these changes afford some measure of the degree of hypoxia in shock, they do not provide a reliable prognosis of death or survival.

Wiggers *et al.* (22) and Remington *et al.* (14) have demonstrated that small doses of Dibenamine administered to dogs shortly before or after hemorrhage result in a significant increase in the survival rate when the rate is compared to that of untreated controls. The latter authors, however, failed to elucidate the mechanism whereby Dibenamine protected the life of the animal. In a preliminary report we observed that the increased survival in Dibenamine-

[1] *Manuscript received April 22, 1955.*
Contribution from the Department of Physiology, University of Western Ontario, London, Ontario. This investigation was supported by Grant 298 from the Defence Research Board of Canada.
[2] *Present address: Collip Medical Research Laboratory, University of Western Ontario, London, Ontario.*
[3] *Present address: Department of Pharmacology, University of Michigan, Ann Arbor, Michigan, U.S.A.*

treated animals was related to a significantly lower peripheral resistance which resulted in a significantly greater blood flow, oxygen consumption, and arterial oxygen transport (5).

The present work deals mainly with (1) the effect of the administration of Dibenamine or Dibenzyline on the survival, plasma levels of amino acid nitrogen, lactic acid, pyruvic acid, lactate to pyruvate ratio, bicarbonate content, pH, and total arterial oxygen transport of the dog in hemorrhagic, hypotensive shock; (2) the value of these indices as indications of the severity of shock and eventual survival.

Methods

Healthy mongrel dogs (10–25 kgm.) were used throughout this investigation. The animals were allowed water ad libitum but were deprived of food for 18 hr. before the experiment. They were anesthetized with approximately 30 mgm./kgm. of sodium pentobarbital given intravenously *two hours* before the period of control observations was begun. The shock induction procedure was a modification of Wiggers' technique (22) and has been described in detail elsewhere (4). Briefly, the method consisted of bleeding the animal from the femoral artery into an elevated Lamson-type reservoir so that a blood pressure of 43–45 mm. Hg was reached in 10 min. time. This hypotensive blood pressure was maintained for exactly 90 min. with the reservoir open to the animal at all times. Accurate control of the blood pressure within this narrow limit was maintained either by raising or lowering the reservoir bottles, or, as in some cases where this was not sufficient, by the gentle propulsion of blood to and from the animal and the reservoir. The brachial artery blood pressure was recorded throughout with a mercury manometer and kymograph. At the end of exactly 90 min. of hypotension all the withdrawn blood contained in the reservoir (except that taken for analytical purposes) was reinfused by way of the femoral artery. This was accomplished by first raising the reservoir to a height of 100 cm. above table level and allowing it to remain there for five minutes. Reinfusion of the remaining blood was then completed by raising the reservoir to a height equivalent to 150 mm. Hg pressure. To prevent clotting of blood in the reservoir 5 mgm./kgm. of heparin in 10 ml. of isotonic saline was administered slowly into the animal by way of the right atrial catheter five minutes before bleeding was begun.

Control samples of blood were drawn 60 min. before and again just before hemorrhage. The prehemorrhage value of each index shown in the tables is an average of these two determinations. During the hypotensive period blood samples were withdrawn 45, 75, and 95 min. after the beginning of hemorrhage, and in the postinfusion period one and five hours after the beginning of reinfusion.

Arterial plasma amino nitrogen was measured according to the method of Frame *et al.* (10) and Russell (15). Plasma lactic acid was determined by the method of Barker and Summerson (2), and plasma pyruvic acid by the method Friedemann and Haugen (11*a*).

Plasma bicarbonate content was estimated from the pH and plasma carbon dioxide content by means of the line chart devised by Van Slyke and Sendroy (21). Arterial blood pH was determined with a Leeds and Northrop glass electrode pH meter within seconds of sampling and with minimal agitation. Oxygen consumption was recorded continuously either with a Sanborn Metabulator or a Benedict-Roth Metabolism apparatus containing room air. The partial pressure of oxygen within the bellows or container was maintained equivalent to that of room air by the constant infusion of 100% oxygen in amounts equivalent to those taken up by the animal (4). The cardiac output was calculated by the direct Fick method, blood oxygen, and carbon dioxide content being determined by the manometric method of Van Slyke and Neill (20). Total arterial oxygen transport was calculated by the formula:

$$\begin{matrix}\text{arterial oxygen transport} \\ \text{(cc./kgm./min.)}\end{matrix} = \begin{matrix}\text{(cardiac output ml./kgm./min.)} \\ \text{(arterial oxygen content in vol. \%)}\end{matrix}.$$

The results were treated by standard statistical methods (1). The significance of difference between means was determined with Student's "*t*" test.

Four experimental groups were studied:

(1) The untreated control group.

(2) The group given 4 mgm./kgm. of Dibenamine hydrochloride (N,N-dibenzyl-β-chlorethylamine hydrochloride) in 10 ml. of saline, administered over a 10-min. period by way of the right atrial catheter, *85 min.* after the onset of hemorrhage.

(3) The group given 0.8 mgm./kgm. of Dibenzyline hydrochloride (N-phenoxyisopropyl-N-benzyl-β-chlorethylamine hydrochloride) in the same manner *85 min.* after the onset of hemorrhage.

(4) The group given 4 mgm./kgm. of Dibenamine in the same manner *30 min.* after the onset of hemorrhage.

The criterion of survival was that the animals live 96 hr. after the beginning of reinfusion.

Results

I (a). Comparison of Untreated and 'Early-Dibenamine-treated' Animals

Of the 15 untreated animals, 10 died and 5 survived. The mean survival time of the dying animals was 19.0 hr. (range 1–90 hr). Of the six animals treated with Dibenamine 30 min. after the onset of hemorrhage, five survived and one died. The survival time for the dying animal was 65 hr. following reinfusion.

Table I shows that the mean increase in plasma amino nitrogen during the hypotensive period was significantly less in the Dibenamine-treated than in the untreated group as was the percentage decrease in total arterial oxygen transport. One hour after reinfusion both the plasma amino nitrogen and the arterial oxygen transport had returned to or risen above their prehemorrhage values in the treated group. On the other hand, in the untreated animals the mean plasma amino nitrogen level was probably significantly greater ($P < .05$)

TABLE I

EFFECTS OF EARLY TREATMENT IN HEMORRHAGIC HYPOTENSIVE SHOCK

Index	Prehemorrhage period	Hypotensive period (min. after onset of hemorrhage) 45	75	95	Postinfusion period (hr. after reinfusion) One	Five
Untreated group						
P.A.N.	5.7±0.21 (15)	3.5±0.47 (15)	5.6±0.57* (13)	6.0±0.62† (14)	3.8±0.99* (14)	1.6±0.51 (11)
O_2 trans.	39.6±3.78 (14)	15.7±1.68* (14)	16.7±1.82* (12)	16.8±1.66† (14)	68.6±6.12† (13)	—
Dibenamine treated group						
P.A.N.	5.8±0.58 (6)	2.2±0.75 (6)	2.6±0.87* (4)	1.9±0.71† (6)	0.2± 0.59* (6)	0.2±0.78 (4)
O_2 trans.	37.8±5.60 (6)	23.5±3.29* (6)	27.1±4.98* (4)	32.1±4.45† (6)	113.3±19.8† (6)	—

* $P < .05$.
† $P < .01$.

LEGEND FOR TABLES I–V

1. *All values are shown as mean ± S.E.M.*
2. *( ) Number of animals in group.*
3. *P.A.N. (plasma amino nitrogen), L.A. (lactic acid), and P.A. (pyruvic acid)—prehemorrhage values are expressed in mgm./100 ml. plasma—hypotensive and postinfusion values as mgm./100 ml. mean increase above respective prehemorrhage value.*
4. *L/P ratio—hypotensive and postinfusion values are the mean increases above prehemorrhage value.*
5. *pH of plasma—absolute units throughout.*
6. *$BHCO_3$ (plasma bicarbonate content) prehemorrhage value is expressed in mM./l.—hypotensive and postinfusion values are mean decreases in mM./l. from prehemorrhage value.*
7. *O_2 trans. (arterial blood oxygen transport)—prehemorrhage value in cc./kgm./min.: hypotensive and postinfusion values expressed as percentage of prehemorrhage value.*

while the arterial oxygen transport (still only 68.6 ± 6.12% of its prehemorrhage value) was significantly less than in the treated group one hour after reinfusion. No further change in plasma amino nitrogen was observed in the treated group five hours after reinfusion, while in the untreated group a slow decline toward a normal level continued.

I (b). Comparison of Untreated, 'Late-Dibenamine-treated', and Dibenzyline-treated Groups

The administration of Dibenamine or Dibenzyline 85 min. after the onset of hemorrhage did not result in an increased survival rate over that of the untreated animals (Table II). Although the mean survival time of the dying animals was longer in the Dibenzyline-treated group than in the Dibenamine-treated group, this difference in survival time is not significant.

TABLE II

EFFECT OF LATE TREATMENT ON SURVIVAL AND ITS DURATION IN HEMORRHAGIC HYPOTENSIVE SHOCK

	No. of dogs	Survived	Died	Mean survival time, hr. after reinfusion
Untreated group	8	4	4	15.5±8.22
Dibenamine-treated	9	4	5	13.5±6.99
Dibenzyline-treated	8	4	4	33.0±7.59

The metabolic changes in these experimental groups are shown in Table III. The plasma amino nitrogen, lactic acid, pyruvic acid, and L/P ratio rose progressively in all three groups and reached their highest values just before reinfusion. At the same time the plasma bicarbonate content and pH showed a steady decline. No significant difference between the treated and the untreated could be demonstrated in these indices at this time or in the post-infusion period, although the oxygen transport had then returned to prehemorrhage values in the treated groups.

I (c). Comparison of the Late-treated Dibenamine and Dibenzyline Survivors and Fatalities

Since Dibenamine and Dibenzyline are analogues and are considered to have a qualitatively similar pharmacological action, the survivors and fatalities of the two groups treated with these drugs have been combined for statistical comparison. The results are shown in Table IV. The plasma amino nitrogen was the only index measured in which significant differences appear between the survivors and fatalities during the hypotensive and postinfusion periods. The increase in plasma amino nitrogen was less in the survivors, even before administration of the drugs and despite the fact that the oxygen transport was almost identical in the two groups.

TABLE III

EFFECTS OF LATE TREATMENT IN HEMORRHAGIC HYPOTENSIVE SHOCK

Index	Prehemorrhage period	Hypotensive period (min. after onset of hemorrhage)			Postinfusion period (hr. after reinfusion)	
		45	75	95	One	Five
Untreated group						
P.A.N.	5.8±0.33 (8)	3.1± 0.71 (8)	5.4± 0.75 (8)	5.5± 1.00 (7)	3.2± 0.99 (8)	1.5± 0.75 (7)
L.A.	16.8±2.00 (8)	60.0±10.7 (8)	99.0±11.6 (8)	113.0±13.2 (8)	44.0± 5.8 (7)	21.6±13.8 (6)
P.A.	1.6±0.15 (8)	1.4± 0.46 (8)	1.6± 0.46 (8)	1.5± 0.33* (7)	2.3± 0.30† (7)	0.7± 0.63 (6)
L/P ratio	11.2±1.43 (8)	20.3± 7.63 (8)	31.6± 8.06 (8)	37.5± 8.86 (7)	3.7± 0.78 (7)	5.0± 2.40 (6)
pH	7.3±0.02 (7)	7.3± 0.04 (7)	7.2± 0.06 (7)	7.1± 0.05 (7)	7.3± 0.03 (6)	—
$BHCO_3$	21.5±1.56 (6)	−9.1± 1.74 (6)	−12.9± 2.01 (6)	−13.8± 2.20 (6)	−6.1± 1.34 (6)	—
O_2 trans.	34.5±4.88 (8)	19.3± 4.49 (8)	20.8± 4.98 (8)	17.0± 2.67 (7)	69.7± 6.36 (7)	—
Dibenamine-treated group						
P.A.N.	5.6±0.63 (8)	4.1± 1.13 (8)	6.8± 1.24 (8)	7.2± 1.28 (8)	5.4± 1.40 (8)	2.7± 1.58 (7)
L.A.	13.6±1.90 (7)	81.0±12.3 (6)	105.0±13.7 (7)	124.0±14.9 (7)	71.0±11.7 (7)	36.9±12.1 (6)
P.A.	1.4±0.13 (7)	1.0± 0.19 (6)	1.5± 0.13 (7)	2.6± 0.22* (7)	4.5± 0.57† (7)	2.2± 0.31 (6)
L/P ratio	10.0±1.03 (7)	30.5± 5.51 (6)	31.0± 4.74 (7)	24.1± 2.94 (7)	5.0± 1.94 (7)	4.1± 1.55 (6)
pH	7.4±0.03 (7)	7.2± 0.05 (7)	7.1± 0.05 (7)	7.0± 0.05 (7)	7.2± 0.05 (5)	—
$BHCO_3$	23.1±0.86 (7)	−13.3± 0.49 (7)	−16.1± 1.17 (7)	−17.1± 0.93 (7)	−9.1± 1.17 (5)	—
O_2 trans.	29.2±3.00 (9)	18.5± 2.64 (9)	19.5± 2.29 (9)	29.9± 3.73 (9)	115·0± 5.47 (9)	—
Dibenzyline-treated group						
P.A.N.	5.9±0.56 (8)	3.4± 1.08 (8)	5.0± 1.20 (8)	4.6± 1.14 (8)	2.6± 1.02 (7)	0.7± 0.40 (8)
L.A.	13.1±1.77 (8)	67.0± 9.30 (8)	93.9± 7.40 (8)	98.3± 6.18 (8)	47.6± 9.79 (8)	5.3± 4.28 (8)
P.A.	1.3±0.17 (8)	1.36± 8.25 (8)	1.5± 0.20 (8)	1.7± 0.16 (8)	2.7± 0.31 (8)	0.2± 0.28 (8)
L/P ratio	9.9±0.70 (8)	19.9± 4.49 (8)	28.1± 2.57 (8)	28.1± 2.85 (8)	5.2± 1.68 (8)	3.1± 2.56 (8)
pH	7.4±0.02 (7)	7.3± 0.34 (7)	7.2± 0.04 (7)	7.1± 0.04 (6)	7.3± 0.02 (6)	—
$BHCO_3$	21.4±1.17 (7)	−9.2± 1.16 (7)	−12.7± 1.00 (7)	−12.1± 0.61 (6)	−5.1± 0.79 (6)	—
O_2 trans.	29.9±2.45 (8)	18.8± 2.15 (8)	18.3± 1.48 (8)	32.2± 2.95 (8)	105.1± 8.48 (8)	—

* $P < .02$.
† $P < .01$.
See footnote to Table I.

TABLE IV

COMPARISON OF CHANGES IN LATE TREATED SURVIVORS AND TREATED FATALITIES

Index	Prehemorrhage period	Hypotensive period (min. after onset of hemorrhage)			Postinfusion period (hr. after reinfusion)	
		45	75	95	One	Five
Treated survivors						
P.A.N.	6.0±0.76 (7)	2.5± 0.57 (7)	3.6± 0.80* (7)	3.2± 0.59† (7)	1.4± 0.36* (6)	0.1± 0.38† (7)
L.A.	12.1±1.99 (6)	66.6± 8.05 (6)	98.2± 9.76 (6)	106.3±14.30 (6)	48.9±10.36 (6)	7.4± 3.47 (6)
P.A.	1.2±0.14 (6)	1.4± 0.24 (6)	1.5± 0.10 (6)	2.2± 0.28 (6)	2.7± 0.43 (6)	0.7± 0.34 (6)
L/P ratio	10.4±0.95 (6)	21.8± 4.82 (6)	32.0± 4.25 (6)	26.2± 2.67 (6)	5.2± 2.00 (6)	0.2± 1.19 (6)
pH	7.4±0.02 (7)	7.3± 0.05 (7)	7.2± 0.05 (7)	7.1± 0.07 (6)	7.3± 0.03 (5)	—
$BHCO_3$	22.3±1.18 (7)	−9.3± 1.37 (7)	−13.1± 1.17 (7)	−15.0± 1.18 (6)	−6.1± 0.99 (5)	—
O_2 trans.	28.4±2.64 (8)	20.2± 2.88 (8)	19.5± 2.37 (8)	29.6± 2.83 (8)	112.6± 8.58 (8)	—
Treated fatalities						
P.A.N.	5.4±0.45 (9)	4.8± 1.19 (9)	7.6± 1.12* (9)	8.0± 1.07† (9)	5.9± 1.22* (9)	3.2± 1.19‡ (8)
L.A.	14.2±1.65 (9)	74.8±11.80 (8)	100.0±10.30 (9)	111.7±10.76 (9)	64.5±11.29 (9)	28.1±10.06 (8)
P.A.	1.5±0.14 (9)	1.0± 0.22 (8)	1.6± 0.19 (9)	2.1± 0.19 (9)	4.1± 0.54 (9)	1.3± 0.55 (8)
L/P ratio	9.6±0.78 (9)	26.5± 5.46 (8)	27.7± 3.20 (9)	26.3± 3.04 (9)	5.2± 1.57 (9)	5.2± 2.52 (8)
pH	7.4±0.03 (7)	7.2± 0.04 (7)	7.04±0.03 (7)	7.0± 0.02 (7)	7.2± 0.03 (6)	—
$BHCO_3$	22.2±1.11 (7)	−13.1± 1.48 (7)	−15.7± 1.01 (7)	−14.7± 1.40 (7)	−7.6± 1.47 (6)	—
O_2 trans.	30.6±2.84 (9)	17.3± 1.91 (9)	18.5± 1.61 (9)	32.2± 3.79 (9)	109.0± 5.88 (9)	—

* $P = <.02$.
† $P = <.01$.
‡ $P = <.05$.
See footnote to Table I.

TABLE V

COMPARISON OF CHANGES IN UNTREATED SURVIVORS AND UNTREATED FATALITIES

Index	Prehemorrhage period	Hypotensive period (min. after onset of hemorrhage)			Postinfusion period (hr. after reinfusion)	
		45	75	95	One	Five
Untreated survivors						
P.A.N.	6.0±0.38 (5)	2.7±0.91 (5)	5.9±1.08 (4)	6.0±1.01 (5)	1.4±0.83* (5)	0.4±0.62† (5)
O_2 trans.	44.3±5.62 (5)	12.1±1.47 (5)	13.3±1.19 (4)	14.6±1.29 (5)	75.8±8.62 (5)	—
Untreated fatalities						
P.A.N.	5.6±0.28 (10)	3.9±0.54 (10)	5.5±0.82 (9)	6.0±0.84 (9)	5.2±1.30* (9)	2.6±0.51† (6)
O_2 trans.	34.2±3.76 (9)	17.7±2.26 (9)	18.5±2.47 (8)	18.1±2.44 (9)	65.5±7.37 (8)	—

* $P < .1$.
† $P < .05$.
See footnote to Table I.

I (d). Comparison of the Untreated Survivors and Fatalities

The results of a statistical comparison of the untreated survivors and fatalities are shown in Table V. No significant difference during the hypotensive period is apparent between the two groups in either the mean plasma amino nitrogen increase or in the percentage decrease in total arterial oxygen transport. One hour after reinfusion the survivors showed a rapid decrease in the plasma amino nitrogen level from the high values observed just before reinfusion. In the fatalities the decrease was much more gradual. The difference in mean values, however, was not significant ($P < 0.1$). In both the survivors and fatalities the total arterial oxygen transport one hour after reinfusion had shown a sharp increase from the values observed just before reinfusion, but it was still 25 to 35% below the respective prehemorrhage levels. Five hours after reinfusion the mean plasma amino nitrogen level in the fatalities was still 2.6 ± 0.51 mgm.%, while that in the survivors was only 0.42 ± 0.62 mgm.% above the prehemorrhage values ($P < .05$).

Discussion

We have previously reported (5) that treatment with Dibenamine 30 min. after the onset of severe hemorrhage resulted in a better survival rate. This confirms the findings of Wiggers *et al.* (22) and Remington *et al.* (14). We have also (5) shown that this early treatment after hemorrhage inhibited the development of the progressive compensatory vasoconstriction which follows hemorrhage. The lower total peripheral resistance in the treated animals results in a smaller "secondary bleeding volume" (4), and a significantly greater total blood flow, oxygen consumption, per cent oxygen saturation of mixed venous blood, and total arterial oxygen transport. This favorable hemodynamic state in the treated group could explain the increased survival rate and also the lower plasma amino nitrogen. It is not known whether the smaller increase in plasma amino nitrogen in the treated group is the result of a greater ability of the liver to deaminate amino acids or of a smaller production of amino acids in the peripheral tissues. Both these factors are dependent on the adequacy of blood flow and the relative degrees of hypoxia in these sites. Brandfonbrenner and Geller (7) have shown that Dibenamine increases renal blood flow in hemorrhagic hypotensive shock. We do not know in what degree excretion by the kidney was responsible for the difference in plasma amino nitrogen between the Dibenamine-treated and the untreated groups but glomerular filtration is unlikely at a systemic blood pressure of 45 mm. Hg.

The protective effect of sympathetic blocking agents when they are given soon after hemorrhage is brought about by their inhibition of reflex vasoconstriction and, therefore, of secondary bleeding. Clinically, such an effect could be of value when secondary bleeding is a serious threat but only if the means are available to counteract the fatal hypotension which might result from too great an inhibition of vasoconstriction.

There was no difference in survival rate between the untreated animals and those treated with Dibenamine or Dibenzyline 85 min. after the onset of

hemorrhage (Table II). Despite the fact that Beck (4) has shown that the oxygen consumption, cardiac output, and total arterial oxygen transport are definitely improved after the administration of these agents, this hemodynamic improvement does not manifest itself in a significant improvement in the metabolic status (see Table III). In the light of the present results we are forced to conclude that Dibenamine and Dibenzyline administered late in the hypotensive period are ineffective in reversing an otherwise irreversible state. On the other hand, five hours after reinfusion the mean duration of survival is somewhat greater and the general metabolic picture is slightly better in the Dibenzyline-treated group than in the controls. This suggests that this agent, although not adequate even with the replacement of the blood loss at this time, may improve the situation and that it would perhaps be beneficial in conjunction with other therapy.

On examining the problem more closely, we find that metabolic differences do exist when, in the late-treated groups, the survivors are compared with the fatalities (see Table IV). The most striking difference between these two groups is the trend in plasma amino nitrogen during the hypotensive period. It presents a picture comparable to that observed between the group treated early with Dibenamine and the untreated group. In the present case this difference between survivors and fatalities cannot be explained as a result of treatment, since it was present long before the drugs were administered. There is no indication from our study that the eventual survivors were in a better hemodynamic state, as evidenced by the total arterial oxygen transport. The liver, according to Bollman, Mann, and Magath (6), is probably the main site of oxidative deamination of amino acids. Our results would indicate that this function of the liver had been less seriously impaired in those animals which eventually survived the shocking procedure. Maintenance of hepatic blood flow and oxygenation by aortic-portal fistula (8) or viviperfusion (19) have been shown to allow survival in a hemorrhagic shock which would be fatal to a normal animal. The lower plasma amino nitrogen in the survivors may thus reflect a more adequate hepatic blood flow and oxygenation. The mechanism whereby such a preferential redistribution of blood from less essential to more vital organs may take place remains obscure.

The lack of significant differences in plasma amino acid nitrogen between the survivors and fatalities of the untreated group during the hypotensive period makes one question the prognostic value of this index during hypotension (see Table V). The rate at which the plasma amino nitrogen returns toward the normal level after reinfusion is perhaps more valuable for prognosis of survival. The persistence of a high intrahepatic resistance to portal flow in the postinfusion period has been established in the dog by Wiggers *et al.* (23) and Friedman *et al.* (11*b*) and also recently in the rat by Johnson (11*c*). It may be that the amino acid level in the postinfusion period is dependent on the rate at which hepatic blood flow and oxygenation return toward normal levels. This may be one of the decisive and immediate factors determining the final outcome in hemorrhagic shock after transfusion in the dog.

Summary

1. The ability of Dibenamine and Dibenzyline to improve survival rate was investigated in dogs subjected to a standardized shocking procedure (Wiggers) in which, by bleeding into a reservoir, a systemic blood pressure of 43–45 mm. Hg was maintained for 90 min.

2. No difference in survival rate was found between those treated intravenously, 85 min. after the onset of hemorrhage, with Dibenamine (4/9) or Dibenzyline (4/8) and those animals receiving no adrenergic blocking agent (4/8).

3. Neither during the hypotensive period nor after reinfusion did these three groups reveal any significant differences in plasma amino nitrogen, lactic acid, L/P ratio, bicarbonate content, and blood pH. The total arterial oxygen transport, however, was significantly greater in the treated groups after treatment and in postinfusion period. The plasma pyruvate level *after* the administration of Dibenamine rose to values which were significantly greater than those of either the untreated or the Dibenzyline-treated groups.

4. In the two late-treated groups, the only significant difference between survivors and fatalities was the smaller increase in plasma amino nitrogen shown by the survivors during both the hypotensive and postinfusion periods. This suggests that hepatic embarrassment may have been less severe in the survivors, perhaps as a result of preferential rerouting of blood from nonessential areas to the liver.

5. A significant increase in survival rate over that of untreated controls was obtained when Dibenamine was administered relatively soon (30 min.) after the onset of hemorrhage (5/15 vs. 5/6). The increase in plasma amino nitrogen and the decrease in total oxygen transport were significantly less during the period of controlled hypotension in the Dibenamine-treated than in the untreated animals. Five hours after reinfusion the plasma amino nitrogen in the treated group had returned to the prehemorrhage value, while in the untreated group it was considerably above its prehemorrhage level.

6. No significant difference in plasma amino nitrogen and arterial oxygen transport during the hypotensive period could be demonstrated when the survivors of the untreated groups were compared with the fatalities of that group. During the postinfusion period, although no significant difference between the untreated survivors and fatalities was observed in either total arterial oxygen transport or plasma amino nitrogen, the rate at which plasma amino nitrogen returned toward prehemorrhage levels was faster in the surviving animals than in those which later died.

Acknowledgments

The authors acknowledge the help of Dr. C. W. Gowdey in the design and conduct of this study and of Dr. J. W. Pearce in the later phases. Mrs. P. D. Marchuk gave valuable technical assistance throughout the investigation. We are most grateful to Smith, Kline, and French Co., Philadelphia, for the generous supply of Dibenamine and Dibenzyline used in this study.

References

1. ARKIN, H. and COLTON, R. R. An outline of statistical methods. 4th ed. Barnes & Noble, Inc., New York. 1949.
2. BARKER, S. B. and SUMMERSON, W. H. J. J. Biol. Chem. 138 : 535. 1941.
3. BEATTY, C. H. Am. J. Physiol. 143 : 579. 1945.
4. BECK, L. Ph.D. Thesis, Univ. of Western Ontario, London, Ont. 1953.
5. BECK, L. and LOTZ, F. Federation Proc. 12 : 13. 1953.
6. BOLLMAN, J. L., MANN, F. C., and MAGATH, T. B. Am. J. Physiol. 78 : 258. 1926.
7. BRANDFONBRENNER, N. and GELLER, H. M. Am. J. Physiol. 171 : 482. 1952.
8. COHN, R. and PARSONS, H. Am. J. Physiol. 160 : 437. 1950.
9. ENGEL, F. L. J. Mt. Sinai Hosp., N.Y. 12 : 152. 1945.
10. FRAME, E. G., RUSSELL, J. A., and WILHELMI, A. E. J. Biol. Chem. 149 : 225. 1943.
11(*a*). FRIEDEMANN, T. E. and HAUGEN, G. E. J. Biol. Chem. 147 : 415. 1943.
(*b*). FRIEDMAN, E. W., FRANK, H. A., and FINE, J. Ann. Surg. 134 : 70. 1951.
(*c*). JOHNSON, D. H. J. Physiol. (London), 126 : 413. 1954.
12. KLINE, D. L. Am. J. Physiol. 146 : 654. 1946.
13. NASTUK, W. L. and BEATTY, C. N. Am. J. Physiol. 156 : 202. 1949.
14. REMINGTON, J. W., HAMILTON, W. F., BOYD, G. W., JR., HAMILTON, W. F., JR., and CADELL, H. N. Am. J. Physiol. 161 : 116. 1950.
15. RUSSELL, J. A. J. Biol. Chem. 156 : 467. 1944.
16. RUSSELL, J. A., LONG, C. N. H., and ENGEL, F. L. J. Exptl. Med. 79 : 1. 1944.
17. SELIGMAN, A. M., ALEXANDER, B., FRANK, H. A., and FINE, J. Am. J. Physiol. 152 : 531. 1948.
18. SELIGMAN, A. M., FRANK, H. A., ALEXANDER, B., and FINE, J. J. Clin. Invest. 26 : 536. 1947.
19. SELIGMAN, A. M., FRANK, H. A., and FINE, J. J. Clin. Invest. 26 : 530. 1947.
20. VAN SLYKE, D. D. and NEILL, T. Quantitative clinical chemistry. Vol. 2. The Williams & Wilkins Company, Baltimore, Md. 1932. p. 324.
21. VAN SLYKE, D. D. and SENDROY, J., JR. J. Biol. Chem. 78 : 781. 1928.
22. WIGGERS, H. C., GOLDBERG, H., ROEMHILD, F., and INGRAHAM, R. C. Circulation, 2 : 179. 1950.
23. WIGGERS, C. J., OPDYKE, D. F., and JOHNSON, J. R. Am. J. Physiol. 146 : 192. 1946.

RELIABILITY OF THE RIBOFLAVIN EXCRETION TECHNIQUE IN DETERMINING AVAILABILITY OF COATED TABLETS[1]

By D. G. Chapman and J. A. Campbell

Abstract

Further studies have been carried out to assess the reliability of the human bio-assay technique using the excretion of riboflavin to estimate the availability of sugar-coated tablets. Using eight individuals, it has been shown that for 1, 3, and 5 mgm. amounts of riboflavin the excretion is the same whether these amounts are taken as a single dose or in 10 small doses totalling the same amount. For doses of riboflavin of 1, 3, 5, 7.5, and 10 mgm. a linear relationship was found to exist between the amount ingested and excreted. The equation for this dose response line was found to be $y = 0.580x - 0.610$. The intercept -0.610 was significantly different from zero, indicating that the line did not pass through the origin. The results furnish further evidence of the validity of the urinary excretion of riboflavin for determining the physiological availability of coated tablets.

Introduction

Chapman, Crisafio, and Campbell (3) reported that sugar-coated tablets which did not disintegrate *in vitro* in 60 min. were not completely available to the human subjects as judged by urinary excretion of riboflavin. The absence of extra riboflavin in the urine following the ingestion of a tablet was interpreted as indicating low availability of riboflavin from that particular tablet. This conclusion was criticized (2) on the basis that some tablets might release their riboflavin content to the body at such a rate that it would be utilized as quickly as it was released and hence would not be excreted in the urine; and that therefore low excretion of riboflavin would not necessarily indicate low availability of riboflavin from that tablet.

Since no information seemed available in the literature, a study was carried out to compare the excretion of riboflavin following the ingestion of repeated small amounts with the excretion from the same amount taken in a single dose. The relationship between the ingestion and excretion of riboflavin at five levels of intake has been examined for purposes of calculating the availability of riboflavin from coated tablets.

Procedure

Subjects

Eight male employees of the Food and Drug Laboratories volunteered as subjects for the study. All were shown to be receiving nutritionally adequate amounts of riboflavin as indicated by the excretion of riboflavin. While on test the subjects were permitted to consume their regular meals, but were cautioned to refrain from eating foods such as liver, which are known to be high in riboflavin.

[1] *Manuscript received April 12, 1955.*

Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada.

Assay Periods

During each test-week, urine was collected beginning at 9 a.m. on Monday and continuing until 5 p.m. on Tuesday. During this 32 hr. period, the urine was collected in three lots, the first after an 8 hr., the second a 16 hr., and the third an 8 hr. period. On Wednesday at 9 a.m. a known amount of riboflavin was taken either in a single dose or in repeated small doses and the urine was again collected until 5 p.m. on Thursday. The riboflavin found in the urine collected on Monday and Tuesday served as the basal excretion of riboflavin and when it was subtracted from the riboflavin found in the urine collected on Wednesday and Thursday, the amount of riboflavin excreted from the extra riboflavin ingested was obtained.

Three amounts of riboflavin, 1, 3, and 5 mgm., were taken in a single dose or in 10 small doses spread over the working day. For the small dose a solution of riboflavin buffered to pH 3 was prepared and an aliquot was ingested by the subjects at approximately hourly intervals until 10 doses had been taken during the period from 9 a.m. to 5 p.m. The urine was collected for 24 hr. following the ingestion of the last dose at 5 p.m., or a total of 32 hr. from the beginning of the test. For a single dose, the riboflavin was taken either in the form of a compressed tablet or a solution. In addition to the above amounts, 7.5 and 10 mgm. quantities of riboflavin were taken as a single dose.

Determination of Riboflavin

Riboflavin in the urine, tablets, and solutions was determined by the U.S.P. fluorometric procedure (5).

Results and Discussion

The excretion of riboflavin in the urine following the ingestion of a single dose or repeated small doses totalling the same amount is shown in Table I. It can be seen from the mean excretion of the eight subjects that there was no difference between the single large dose and the repeated small doses. However, there was less variation between individuals when the riboflavin was taken in small amounts. This finding refutes the suggestion that a tablet might release its riboflavin at such a rate that it would be utilized by the body rather than be excreted. Even when 0.1 mgm. of riboflavin was taken each hour for 10 hr., the total riboflavin excreted was the same as when 1.0 mgm. was taken in a single dose. These findings indicate that urinary excretion of riboflavin was not influenced by the rate at which it was released to the body and that it is a reliable method of determining the availability of riboflavin from a coated tablet. Conversely, if no extra riboflavin were excreted after the ingestion of a tablet, it can only mean that no riboflavin was released from the tablet.

It was also noted in Table I that the per cent of the ingested riboflavin which was excreted varied to a certain extent with the dose. This appeared to be at variance with the data reported by Melnick, Hochberg, and Oser (4).

TABLE I

URINARY EXCRETION OF RIBOFLAVIN, OVER A 32-HR. PERIOD, TAKEN AS A SINGLE DOSE OR REPEATED SMALL DOSES

Subject	Riboflavin ingested, mgm.					
	Single dose			Ten small doses		
	1	3	5	1	3	5
	Per cent of dose excreted					
A	10	30	49	23	38	49
B	22	46	73	21	40	70
C	—	49	41	6	37	46
D	27	46	—	27	40	47
E	28	30	21	38	29	44
F	32	47	42	26	32	42
G	9	—	59	24	—	44
H	42	26	—	28	35	—
Mean	24	39	48	24	36	49

TABLE II

URINARY EXCRETION OF RIBOFLAVIN FROM A SINGLE DOSE IN 24 HR.

Subject	Riboflavin ingested, mgm.				
	1	3	5	7.5	10
	Per cent of dose excreted				
A	6	23	49	44	49
B	13	44	70	45	52
C	—	42	35	52	70
D	25	39	—	47	38
E	18	31	21	45	45
F	40	45	43	44	62
G	− 7	—	58	53	57
H	31	28	—	54	52
Mean	18	36	46	48	53

TABLE III

ANALYSIS OF VARIANCE OF THE URINARY EXCRETION OF FIVE DOSAGE LEVELS OF RIBOFLAVIN BY EIGHT SUBJECTS

Source of variation	Degrees of freedom	Mean squares
Between subjects	7	388,407
Over-all regression	1	136,696,256
Non-linearity of over-all regression	3	114,025
Heterogeneity of individual regression	7	724,736
Residual variance	21–4	300,280

It was therefore considered important to determine the relationship between the ingestion of riboflavin and its excretion at other levels of intake. To do this, amounts of riboflavin from 1 to 10 mgm. were ingested as a single dose and the extra amount excreted during the following 24 hr. was determined. The results of this study are shown in Table II and the analysis of variance in Table III. Four missing values were calculated to complete the Table and to facilitate the analysis. The data in Table II indicate that there is a marked difference in the percentage of riboflavin excreted at different levels of intake. It can be seen from Table III that the excretions of riboflavin for the eight subjects are not significantly different. It is also apparent that the five points representing the excretions from the five levels of intake can be specified by a linear equation. However, there is a suggestion of slope differences between subjects. This is evident in the individual slopes for the eight subjects plotted in Fig. 1. The equation for the mean response line was found to be $y = 0.580x - 0.610$. The intercept -0.610 was significantly different from zero indicating that the line did not pass through

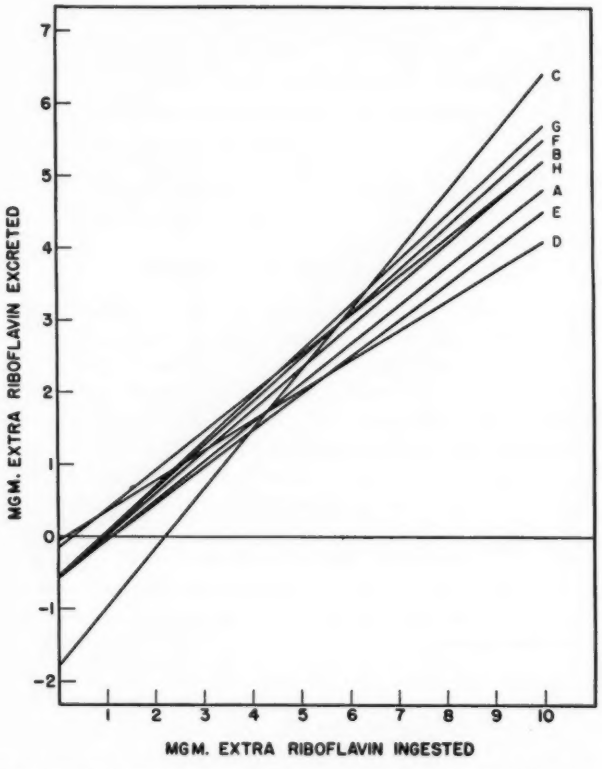


FIG. 1. Relationship between extra riboflavin ingested and excreted for eight subjects.

the origin. This line shows the same relationship between riboflavin ingested and excreted as that reported by Brewer *et al.* (1) for the values to be found in the literature.

Melnick, Hochberg, and Oser (4) have stated that there is a linear relationship between dosage and response and these authors have extrapolated the line to pass through the origin. The results reported here confirm the fact that a linear relationship does exist between the extra riboflavin ingested and the extra riboflavin excreted, but indicate that the response line should not be extrapolated through zero. The slope, 0.580, indicates that over half of each additional quantity of ingested riboflavin was excreted.

In view of the fact that the line relating ingestion and excretion of riboflavin may not pass through the origin, a direct proportional relationship between these two factors does not exist. Thus when calculating the availability of riboflavin from a tablet, one should refer to a curve for each subject such as is shown in Fig. 1 rather than use the technique of Melnick *et al.* (4) which assumes that there is a constant proportional relationship between ingestion and excretion at all levels.

The effect of this approach was studied on the data reported by Chapman, Crisafio, and Campbell (3). Of the 25 tablets studied, 10 were reported to be incompletely available as calculated by the technique of Melnick *et al.* (4). When the data on these 10 tablets were recalculated using the curves shown in Fig. 1, their apparent per cent availability increased, but still did not reach the 70% level which was considered to be the borderline between unsatisfactory and satisfactory availability. Obviously the difference between the two procedures becomes less in the range of 5 to 10 mgm. of riboflavin.

The results of this study have confirmed the conclusions of Melnick, Hochberg, and Oser (4) and Chapman, Crisafio, and Campbell (3) that the urinary excretion of riboflavin is a valid and reliable procedure for determining the physiological availability of coated tablets.

Acknowledgments

The authors are indebted to Mr. N. T. Gridgeman of the National Research Council for advice regarding the statistical treatment of the results, to Mr. R. Dufault for riboflavin analyses, and to those members of the staff of the Food and Drug Laboratory who kindly consented to act as subjects.

References

1. Brewer, W., Porter, T., Ingalls, R., and Ohlson, M. A. J. Nutrition, 32 : 583. 1946.
2. Canadian Pharmaceutical Manufacturers Association. Private communication.
3. Chapman, D. G., Crisafio, R., and Campbell, J. A. J. Am. Pharm. Assoc. Sci. Ed. 43 : 297. 1954.
4. Melnick, D., Hochberg, M., and Oser, B. L. J. Nutrition, 30 : 67. 1945.
5. The United States pharmacopoeia. 14th revision. Mack Printing Company, Easton, Pa. 1950.

SKIN METABOLISM IN RATS EXPOSED TO COLD[1]

By André DesMarais[2]

Abstract

In a study of skin metabolism, it has been found that the activity of the succinic dehydrogenase in this tissue is increased during exposure to cold and that this increase is prevented by administration of sodium ascorbate.

The role of the skin in physical thermoregulation has been studied quite extensively. The skin has always been considered as the site of heat exchange through vasomotor changes and, as such, its role was considered as merely physical. Changes in skin temperature during exposure to cold would be dependent mostly on variations in peripheral circulation and, to a lesser degree, on the thickness of subcutaneous fat layers (5, 6). Although these factors are not only important but actually determinant as far as skin temperature is concerned, nothing in the literature indicates whether continuous exposure to cold leads to changes in endogenous skin metabolism.

Material and Methods

The experimental animals used were male albino rats of the Wistar strain, weighing 160 to 180 gm. They were divided into seven groups. The animals of groups I*a* and I*b* were given sodium ascorbate for 10 and 12 days, respectively, and all were kept at a temperature of $24.0 \pm 1.5°$ C. In group II*a*, the animals were given ascorbate for 10 days and exposed to cold ($+ 2.0 \pm 1.0°$ C.) for the last two days of the treatment period, while the animals of group II*b* were treated with ascorbate for 12 days and exposed to cold for the last four days of the treatment period. In groups III*a* and III*b*, the animals were exposed to cold for two and four days respectively without treatment with sodium ascorbate. Finally, in group IV, 20 animals were used as absolute controls; they were kept at 24° C. without sodium ascorbate.

The treatment with sodium ascorbate consisted of a total daily dose of 150 mgm. administered orally in two doses of 75 mgm. each. All animals were fed ad libitum Purina fox chow checkers and tap water.

To follow the changes in the endogenous metabolism of the skin, the activity of the succinic dehydrogenase (SD) was determined by a technique adapted from the method described by Perry and Cumming (9) for the adrenals.

Contiguous strips of skin, less than 1 mm. in width and weighing between 80 and 120 mgm., were cut from a shaved area of the back of the animal along the median line and at the height of the kidney. From one to two strips were used for total nitrogen and SD determinations, respectively. Nitrogen was determined by the micro-Kjeldahl method with the use of

[1] *Manuscript received April 25, 1955.*
Contribution from the Department of Experimental Physiology, Laval University, Quebec, Que. This work was supported by a grant of the Defence Research Board of Canada.
[2] *Research Assistant.*

Conway cells (7). The method of Perry and Cumming (9) was adapted to the determination of SD in the skin as follows. After a one hour incubation in the usual medium, the strips were blotted on filter paper and immersed in 2 ml. of acetone for three hours in a graduated centrifuge tube for complete extraction of the reduced triphenyl-tetrazolium chloride (TTC). The skin strips were then removed and washed over the tube with 1 ml. of acetone; the acetone was dried with at least three grams of sodium sulphate. Following centrifugation, the volume of acetone was completed to 5 ml. and the color read in a Beckman DU spectrophotometer. The results are expressed as micrograms of reduced TTC per hundred mgm. of nitrogen.

TABLE I

EFFECTS OF EXPOSURE TO COLD AND SODIUM ASCORBATE TREATMENT ON THE SUCCINIC DEHYDROGENASE ACTIVITY OF THE SKIN

Treatment		Group	Number of animals	Skin SD activity, μgm. TTC per 100 mgm. N	Diff. from abs. control	*t* values
Ascorbate	Cold					
10 days	None	I*a*	10	2.25 ± 0.32	0.36 ± 0.34	1.05
10 days	2 days	II*a*	10	1.97 ± 0.11	0.08 ± 0.17	0.47
None	2 days	III*a*	10	2.44 ± 0.09	0.55 ± 0.16	3.43
12 days	None	I*b*	10	1.98 ± 0.09	0.09 ± 0.16	0.56
12 days	4 days	II*b*	9	1.97 ± 0.12	0.08 ± 0.18	0.44
None	4 days	III*b*	9	2.32 ± 0.08	0.43 ± 0.15	2.87
Absolute controls		IV	20	1.89 ± 0.13	—	—

Results

The results of this experiment are shown in Table I and may be summarized as follows:

(*a*) ascorbate administration has no effect on the SD activity at room temperature (groups I*a* and I*b*);

(*b*) exposure to cold enhances the SD activity in the skin of the untreated animals (groups III*a* and III*b*);

(*c*) ascorbate administration during exposure to cold prevents the activation of the SD (groups II*a* and II*b*).

Discussion

It is interesting to note that the increase in succinic dehydrogenase activity of skin tissue during exposure to cold is of the same order of magnitude as that observed in the liver by DesMarais (2), and also as the increase in oxygen consumption observed by Sellers (10) and DesMarais (1). The double parallelism between the increase in succinic dehydrogenase activity of the skin and that of the liver on the one hand, and between the increase in succinic dehydrogenase activity and oxygen consumption of the liver, on the other

hand, is very suggestive. In the first place, the skin, as well as the liver, might be considered as a site of heat production; secondly, the increase in succinic dehydrogenase activity might well be associated with an increase in metabolic rate of the skin. Considering that the weight of the skin exceeds that of the liver (8), the possible contribution of the skin to extra heat production in the cold might not be inconsequential.

The inhibitory effect of ascorbate on the increase in succinic dehydrogenase activity of the skin in a cold environment may be related to the beneficial action of this vitamin in animals exposed to cold, as reported by Dugal (3, 4). As far as changes in succinic dehydrogenase activity may be considered as indicating a change in metabolic rate, this paradoxical effect of ascorbate would lead one to believe that increased skin metabolism upon exposure to cold is possibly not the most economical means of maintaining body temperature.

References

1. DesMarais, A. Rev. can. biol. 12 : 99. 1953.
2. DesMarais, A. Rev. can. biol. 13 : 115. 1954.
3. Dugal, L. P. Trans. of the 2nd Conf. on Cold Injury. Josiah Macy Foundation, N. Y. 1954.
4. Dugal, L. P. Nutrition under climatic stress. Natl. Research Council, Washington, D.C. 1954.
5. Le Blanc, J. Can. J. Biochem. Physiol. 32 : 354. 1954.
6. Le Blanc, J., Stewart, M., Marier, G., and Whillans, M. G. Can. J. Biochem. Physiol. 32 : 407. 1954.
7. Levey, S. Am. J. Pathol. 18 : 435. 1948.
8. Pagé, E. and Babineau, L. M. Can. J. Med. Sci. 31 : 22. 1953.
9. Perry, W. F. and Cumming, G. R. Endocrinology, 50 : 385. 1952.
10. You, R. W. and Sellers, E. A. Endocrinology, 49 : 374. 1951.

THE PREPARATION OF L-α-GLYCERYLPHOSPHORYLCHOLINE FROM LECITHINS[1]

By N. H. Tattrie and C. S. McArthur

Abstract

Investigation of the hydrolysis of phosphatidylcholines (lecithins) in hot aqueous alcohol under the influence of mercuric chloride has shown that glycerylphosphorylcholine is formed and that neither racemization nor migration of the phosphorylcholine moiety occurs. The fatty acids are split off much more rapidly than is choline and as a consequence appreciable amounts of glycerylphosphorylcholine are formed. On the basis of these observations a procedure was devised for the hydrolysis of crude lecithin and the isolation of glycerylphosphorylcholine in a yield of 69%. The product was identified as L-α-glycerylphosphorylcholine by analysis of its cadmium chloride complex, and comparison of its optical rotation with that of the synthetic compound of known configuration. Recovery of the diester from this complex was accomplished through removal of the inorganic salt by ion-exchange resins and the free L-α-glycerylphosphorylcholine was crystallized from 99% ethanol.

Introduction

The metabolism of glycerylphosphorylcholine (GPC) and the role of this diester in lipid metabolism remains to be clarified (5, 22). Biological investigations of this compound have been hampered because relatively large amounts of the pure substance have not been readily available.

In the period 1935 to 1945 several attempts were made to isolate GPC from biological materials (3, 4, 9, 13) and although evidence was obtained which indicated that the diester is present in various plant and animal tissues, in no case was GPC isolated and identified. In 1945, Schmidt and his coworkers (19) successfully isolated a levorotatory α-GPC in a fairly pure form from autolyzed beef pancreas. Later Baer and Kates (1) described an elegant chemical synthesis of L-α-GPC in which D-mannitol is the starting material. They also found that, after especial purification, the α-GPC prepared from pancreatic tissue by the procedure of Schmidt *et al.* exhibited the same rotation as that of the synthetic compound i.e. $[\alpha]_D^{25} = -2.8°$ thus proving that the natural diester possesses the L-configuration.

Although L-α-GPC may be prepared by either of these procedures the first is cumbersome and the product obtained requires so much extra purification that the yields are usually low while the preparation by synthesis is costly, laborious, and may be carried out only by a well trained organic chemist in an especially equipped laboratory. The detection by paper chromatography of glycerylphosphorylethanolamine (GPE) among the products formed on heating cephalin with aqueous mercuric chloride (18) made

[1] *Manuscript received April 1, 1955.*

Contribution from the Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan. This work was carried out with the aid of a grant from the National Research Council of Canada. A report of this work was presented at the Annual Meeting of the Western Regional Group of the Medical Advisory Division of the National Research Council, February 17–19, 1955, Edmonton, Alberta. The material of this communication was incorporated into an application for a patent which was filed in the United States Patent Office, July 19, 1954, under serial number 444,373.

it seem worthwhile to investigate the hydrolysis of lecithin under the influence of this catalyst. The present paper describes this study and provides a method for the preparation of L-α-GPC from egg phospholipids.

Experimental

Optimal Reaction Period

One hundred grams of crude egg phospholipid, containing 70% lecithin, was emulsified in a Waring Blendor with 2000 ml. distilled water. To this emulsion 2000 ml. of 99% ethanol containing 200 gm. mercuric chloride was added and the mixture was refluxed for 58 hr. The rate of formation and degradation of GPC was measured in the above reaction mixture by the removal of samples at various intervals and analysis for water-soluble labile choline ester (GPC) by the following procedure: A 50 ml. sample of the reaction mixture was transferred to a 250 ml. centrifuge cup and shaken with 15 ml. of chloroform. Hydrogen sulphide was passed into the solution until the mercury was completely precipitated. The sample was centrifuged at 2000 r.p.m. (International Centrifuge, Size 2) for 20 min. and aliquots removed from the clear aqueous layer were analyzed for free and total choline (7). The results as shown in Fig. 1 indicate that the maximum amount of GPC is formed at 26 hr.

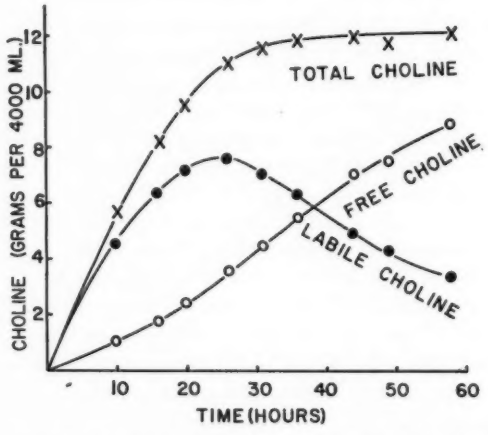


FIG. 1. The formation of water-soluble choline on hydrolysis of lecithin under the influence of mercuric chloride. Total choline fraction contains both free choline and choline liberated when the lipid-free hydrolyzate is boiled with 1.0*N* HCl for 30 min. The difference between total choline and free choline concentrations (labile choline) is assumed to be choline bound as glycerylphosphorylcholine.

Isolation of L-α-*GPC*

A reaction mixture prepared in the same manner as described above was refluxed for 26 hr. and after being cooled the fatty acids and other lipids were removed by extraction with four 600 ml. portions of ethyl ether. Mercury

ions were removed by precipitation with hydrogen sulphide in the presence of barium carbonate followed by filtration. The clear aqueous filtrate was concentrated under reduced pressure at a bath temperature of 45° until the alcohol and ether were distilled off. The yellow aqueous solution was then passed through a mixture of Amberlites IR-45 base (2 volumes) and IRC-50 acid (1 volume). The clear colorless effluent was concentrated under reduced pressure at a bath temperature of 45°. The sirupy residue was dissolved in 500 ml. of 99% ethanol and a saturated solution of cadmium chloride in 95% ethanol was added slowly with stirring until precipitation of the cadmium chloride complex was complete. After the mixture had been allowed to stand in the refrigerator for three hours, the dense white precipitate was filtered off with suction, washed with cold 99% ethanol, washed with ether, and dried over phosphorus pentoxide in a vacuum desiccator. The yield of the amorphous cadmium chloride complex was 34.25 gm. and the over-all yield of product, based on the amount of lecithin in the starting material, was 69.4%. Calculated for $(C_8H_{22}O_7NP)_2\,(CdCl_2)_3$ (1100) : choline (21.9%). Found : (21.4%).

For purposes of identification the amorphous cadmium chloride compound of GPC was converted to the crystalline cadmium chloride compound according to the procedure of Baer and Kates (1). A solution of 12.4 gm. of the amorphous complex in 150 ml. of water was diluted gradually with 600 ml. of 99% ethanol. The clear liquid was kept at room temperature for 12 hr. followed by 12 hr. in the refrigerator at 5°. The crystalline precipitate was filtered with suction and washed with a small volume of cold 80% ethanol. After air-drying the crystals weighed 7.15 gm., (60%). On drying over phosphorus pentoxide *in vacuo* at 56°, the crystalline compound lost 10.8% of its weight. Calculated for a loss of three moles of water 10.92%. The dried product on analysis gave the following data: N, 3.15%; P, 7.02%; Cl, 16.01%; Cd, 25.57%; choline 27.41%. $[\alpha]_D^{25^0}$ −1.33° in water (*c*, 10.16). Calculated for $C_8H_{20}O_6NP\ CdCl_2$ (440.6): N, 3.18%; P, 7.02%; Cl, 16.09%; Cd, 25.52%; choline 27.50%.

The vicinal glycol content of the crystalline cadmium chloride compound was determined by reaction with periodic acid after removal of the cadmium with hydrogen sulphide. This was carried out according to the procedure of Voris, Ellis, and Maynard (23) except that methanolic phenolphthalein was used as an indicator when the reaction mixture was neutralized prior to the addition of phosphate buffer pH 6.0 and titration of the excess of periodate ions. It was found that 0.0885 mM. of the compound consumed 0.0882 mM. of HIO_4 in 30 min. (99.7%).

The analyses and a comparison of the optical rotation with the value $[\alpha]_D^{24} = -1.4°$ (*c*, 5.5) reported by Baer and Kates (1) for the crystalline cadmium chloride complex of synthetic L-α-GPC indicated that the product has the α-structure and L-configuration.

The free diester (L-α-GPC) was recovered from its crystalline cadmium chloride compound in the following manner: an aqueous solution of the

cadmium chloride complex (2%) was passed through a mixture of Amberlites IR-45 (2 volumes) and IRC-50 (1 volume). The clear colorless effluent was concentrated under reduced pressure at a bath temperature of 45°. The sirup was dried further *in vacuo* 56° over phosphorus pentoxide for 48 hr. The vitreous product thus obtained was stored in a vacuum desiccator and after standing for several weeks it became crystalline. Subsequently, it was found that the vitreous material could be crystallized from 99% ethanol by the following procedure: 2.99 gm. of the product were dissolved in 20 ml. of 99% ethanol at 60°. The clear solution was placed in a refrigerator at 5° for three hours and then kept at − 15° for a further period of three hours. The crystals were filtered with suction, washed with cold 99% ethanol and finally with ether. After being dried *in vacuo* at 56° over phosphorus pentoxide the crystalline compound weighed 2.10 gm. (70%); m.p. 142.5–143° with sintering at 141° (short stem thermometer); $[\alpha]_D^{25} = -2.89°$ (*c*, 4.61). On analysis the following results were obtained: Calculated for $C_8H_{20}O_6NP$ (257.2): C, 37.35; H, 7.84; N, 5.45; P, 12.04; ester choline 47.12. Found: C, 37.40; H, 7.77; N, 5.42; P, 12.05; ester choline, 46.89. No free choline could be detected on addition of 2% methanolic ammonium reineckate to 50 ml. of a 0.4% aqueous solution of the compound. It was found that 0.0590 mM. of the compound consumed 0.0588 mM. of HIO_4 in 30 min. indicating that the vicinal glycol content was 99.7% of that demanded by theory.

Discussion

The method described in this paper for the preparation of L-α-GPC appears to be the first published procedure by which the natural diester may be obtained in substance from naturally occurring lecithins. Furthermore, this appears to be the first time that L-α-GPC in the free state has been obtained as a crystalline substance. The L-configuration of the crystalline ester was established by comparison of its specific rotation with that of L-α-glycerylphosphorylcholine synthesized by Baer and Kates (1).

Various attempts to isolate GPC as a reineckate salt from the products obtained on heating soybean phospholipid (20% lecithin) with aqueous mercuric chloride were unsuccessful because the crude sirup was insoluble in ethanol. Assuming that the solubility of the GPC was affected by products from lipids other than lecithin it was deemed advisable to use pure lecithin (8) as a starting material. This yielded a hydrolyzate from which a reineckate could be precipitated in alcoholic solution. When the compound was finally isolated as the crystalline cadmium chloride complex it was identified as that of L-α-GPC. The yield was found to be 85% of that potentially present in the original lecithin. Subsequently it was found that crude egg phospholipids, obtained by acetone precipitation, could be used as a starting material if the reineckate precipitation was replaced by a step involving the removal of cations and anions on a mixed bed deionizing column. In our experience the use of ammonium reineckate always left traces of chromium

in the product which were difficult to remove. Since purified egg lecithin is expensive and time-consuming to prepare, these changes both simplified and made the procedure more satisfactory.

Considerable interest has been shown in the past few years in the metabolic role of L-α-GPC and related compounds since Schmidt and co-workers (21) found significant amounts of this in various animal tissues. Lundquist (16) has shown that GPC is a precursor of free choline in mammalian semen. Schmidt and co-workers (20) observed that GPC accumulated during the incubation of rat intestines. In a recent paper Schmidt and co-workers (22) described a procedure for the determination of glycerylphosphoryl esters in tissues and measured the amounts of GPC in various fresh and autolyzed tissues.

The presence of such an intermediate as GPC in the body may be the result of synthetic as well as hydrolytic reactions. Dawson (6), using radioactive phosphorus as a tracer, obtained evidence which suggested that GPC and GPE are present in the rat liver as intermediates in the catabolism of lecithins and cephalins. Kornberg and Pricer (14, 15) have prepared a soluble enzyme system from liver which is capable of combining two coenzyme A-activated fatty acids and glycerophosphoric acid to form phosphatidic acid. Later they found that phosphorylcholine behaved as a precursor of lecithin and suggested that phosphatidic acid and phosphorylcholine condense with the elimination of a molecule of phosphoric acid. Kennedy (10, 11, 12) using rat liver mitochondria found that radioactive choline was incorporated into the mitochondrial phospholipids. L-α-GPC and radioactive phosphorylcholine did not function as intermediates in this system. Further experimental evidence of a more direct nature is desirable to elucidate the metabolic role of GPC and to ascertain whether it has any significance in the biosynthesis of lecithin.

The simple method described for the preparation of L-α-GPC in good yields from mixed phospholipids should make it possible to incorporate tracer elements biologically into the various moieties of the molecule to give compounds which could be used to investigate the biochemistry of this diester of phosphoric acid. The chemical acylation of GPC is being investigated in this laboratory with a view to synthesis of α-lecithins which are difficult to obtain. The readily available L-α-GPC may prove to be a suitable starting material for the preparation of the important metabolite L-α-glycerophosphoric acid which at present can be prepared only by chemical synthesis from D-mannitol (2) or by enzymatic means (17).

Acknowledgment

The authors wish to thank Dr. Erich Baer of the Banting Institute, University of Toronto, for a generous sample of synthetic L-α-glycerylphosphorylcholine, and Mr. J. A. Baignee, Prairie Regional Laboratory, National Research Council, Canada, for the combustion analyses and the measurement of the optical rotation of the crystalline glycerylphosphorylcholine.

References

1. BAER, E. and KATES, M. J. Am. Chem. Soc. 70 : 1394. 1948.
2. Biochemical preparations. Vol. 2. *Edited by* E. G. Ball, John Wiley & Sons, Inc., New York. 1952. p. 31.
3. CANTONI, G. L. Federation Proc. 6 : 314. 1947.
4. CONTARDI, A. and ERCOLE, A. Arch. sci. biol. (Italy), 21 : 1. 1935.
5. CORNATZER, W. E. *In* Phosphorus metabolism. Vol. II. *Edited by* W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore. 1952. p. 247.
6. DAWSON, R. M. C. Biochem. J. (London), 59 : 5. 1955.
7. GLICK, D. J. Biol. Chem. 156 : 643. 1944.
8. HANAHAN, D. J., TURNER, M. B., and JAYKO, M. E. J. Biol. Chem. 192 : 623. 1951.
9. KAHANE, E. and LEVY, J. Compt. rend. 219 : 431. 1944.
10. KENNEDY, E. P. Federation Proc. 11 : 239. 1952.
11. KENNEDY, E. P. J. Biol. Chem. 201 : 399. 1953.
12. KENNEDY, E. P. J. Biol. Chem. 209 : 525. 1954.
13. KING, E. J. and ALOISI, M. Biochem. J. (London), 39 : 470. 1945.
14. KORNBERG, A. and PRICER, W. E., Jr. Federation Proc. 11 : 242. 1952.
15. KORNBERG, A. and PRICER, W. E., Jr. J. Biol. Chem. 204 : 345. 1953.
16. LUNDQUIST, F. Nature, 172 : 587. 1953.
17. MEYERHOF, O. and GREEN, H. J. Biol. Chem. 178 : 656. 1949.
18. NORMAN, J. M. and DAWSON, R. M. C. Biochem. J. (London), 54 : 396. 1953.
19. SCHMIDT, G., GREENBAUM, L. M., FALLOT, P., WALKER, A. C., and THANNHAUSER, S. J. J. Biol. Chem. 212 : 887. 1955.
20. SCHMIDT, G., HECHT, L., FALLOT, P., GREENBAUM, L. M., and THANNHAUSER, S. J. J. Biol. Chem. 197 : 601. 1952.
21. SCHMIDT, G., HECHT, L., and THANNHAUSER, S. J. Federation Proc. 10 : 244. 1951.
22. SCHMIDT, G., HERSHMAN, B., and THANNHAUSER, S. J. J. Biol. Chem. 161 : 523. 1945.
23. VORIS, L., ELLIS, G., and MAYNARD, L. A. J. Biol. Chem. 133 : 491. 1940.

THE EFFECT OF CORTISONE ON THE FOOD CONSUMPTION OF PREGNANT MICE[1]

By Harold Kalter[2]

Abstract

The amount of food eaten daily by control pregnant mice greatly increased during the latter third of pregnancy. The weight curve of the animals closely followed that of food consumption. Treatment with cortisone caused pregnant animals to eat more and weigh less than the controls did. The effects of cortisone on these phenomena did not immediately cease upon completion of the treatment. The teratogenic effect of cortisone, therefore, cannot be a consequence of decreased food intake.

Introduction

When pregnant mice are injected with cortisone acetate, a certain proportion of the offspring are born with characteristic cleft palates (4). Cleft palates macroscopically indistinguishable from these have been induced in offspring of fasted pregnant mice (5). This latter result made a study of the level of food consumption of cortisone-treated mice desirable. In addition, the conflicting results (7, 8) of the effects of cortisone on the volume of food intake have also made a re-examination of this point pertinent.

Materials and Methods

The animals used were F_1 females from reciprocal crosses between the inbred A/Jax and DBA/1 strains of mice. They were backcrossed to A/Jax males. Three or four females and one or two males occupied each breeding cage. Any female observed to have a copulation plug was removed and placed in a separate cage. The day the plug was found was considered day 0 of the gestation period. The isolated females were weighed daily at approximately 10 a.m., and the amount of food eaten in the preceding 24 hr. was measured by weighing the food left in the food bin and subtracting that amount from the former day's value. All measurements were made to the nearest tenth of a gram. The food was Purina Fox Chow, and water was available at all times. The experimental animals were injected intramuscularly in the flank with 2.5 mgm. of cortisone acetate[3] each morning for four successive days beginning on day 9, 10, 11, 12, or 13 of the gestation period. The time of onset of cortisone treatments was varied in this manner because, for the sake of economy, the same animals were being used to obtain information on the efficacy of cortisone as a teratogen, with gestation time as a variable. Preliminary analysis of the results shows that treatments begun on day 11 produced 82% cleft palate. The detailed analysis of the data, it is hoped, will soon be published.

[1] *Manuscript received February 21, 1955.*
Contribution from the Department of Genetics, McGill University, Montreal, Quebec.
[2] *Postdoctoral Fellow, National Cancer Institute, United States Public Health Service. Present address: Children's Hospital Research Foundation, Cincinnati 29, Ohio.*
[3] *Generously given by Dr. J. H. Laurie, Merck and Co., Ltd., Montreal.*

TABLE I

THE MEAN AMOUNTS OF FOOD EATEN BY, AND THE MEAN WEIGHTS OF, CONTROL, CORTISONE-TREATED, AND POST-TREATMENT PREGNANT MICE ON DIFFERENT DAYS OF THE GESTATION PERIOD

Gestation day	Control		Cortisone-treated			Post-treatment		
	Number	Mean ± S.E.	Number	Mean ± S.E.	P*	Number	Mean ± S.E.	P*
Mean amounts of food, gm.								
0	11	4.61 ± 0.26						
1	14	4.59 ± 0.21						
2	16	4.88 ± 0.18						
3	17	4.98 ± 0.17						
4	17	5.42 ± 0.14						
5	18	5.23 ± 0.18						
6	18	5.49 ± 0.18						
7	28	5.49 ± 0.21						
8	38	5.29 ± 0.21						
9	46	5.22 ± 0.18	3	5.97 ± 0.27	.02–.05			
10	52	5.25 ± 0.13	8	6.01 ± 0.53	.10–.20			
11	48	5.46 ± 0.14	20	6.66 ± 0.23	$\ll$.001			
12	37	5.67 ± 0.11	28	6.32 ± 0.22	.01			
13	34	6.05 ± 0.12	34	6.59 ± 0.20	.02–.05			
14	34	6.10 ± 0.16	27	6.83 ± 0.27	.02	9	6.58 ± 0.56	.40
15	34	6.23 ± 0.15	16	7.18 ± 0.22	$<$.001	17	5.40 ± 0.43	.05–.10
16	34	6.12 ± 0.14	6	6.95 ± 0.35	.02–.05	27	5.48 ± 0.20	.01
17	34	5.69 ± 0.15				32	4.97 ± 0.22	.01
18	31	4.52 ± 0.22				30	3.88 ± 0.27	.05–.10
Total	561	5.47 ± 0.04	142	6.62 ± 0.10	$\ll$.001	115	4.99 ± 0.15	$\ll$.001
Control			310	5.70 ± 0.06		167	5.75 ± 0.08	

Mean weights, gm.								
0	62	25.15 ± 0.34						
1	12	26.50 ± 0.61						
2	20	26.57 ± 0.44						
3	16	26.58 ± 0.70						
4	20	27.85 ± 0.51						
5	12	27.58 ± 0.54						
6	13	27.82 ± 0.52						
7	27	27.91 ± 0.40						
8	39	28.10 ± 0.36						
9	51	27.76 ± 0.41						
10	53	28.82 ± 0.69	3	27.27 ± 0.70	.10			
11	56	29.46 ± 0.42	9	29.20 ± 1.17	.80			
12	42	30.52 ± 0.45	23	29.51 ± 1.42	.05			
13	34	32.02 ± 0.60	29	29.63 ± 0.65	.01			
14	37	33.54 ± 0.69	35	30.81 ± 0.67	$<.01$			
15	32	35.81 ± 0.84	25	31.76 ± 0.89	$>.001$	9	31.04 ± 1.30	.001
16	33	36.33 ± 0.78	15	33.05 ± 1.19	$>.02$	15	32.33 ± 1.41	.01–.02
17	32	38.14 ± 1.00	4	37.03 ± 3.06	.70	26	32.82 ± 0.76	$\ll.001$
18	30	41.19 ± 1.07	2	37.65 ± 8.05	.60–.70	30	34.76 ± 0.77	$\ll.001$
19	25	28.20 ± 0.58				19	24.82 ± 0.69	$<.001$
Total	646	30.41 ± 0.69	145	30.55 ± 0.42	$\ll.001$	99	31.63 ± 0.54	$\ll.001$
Control			349	33.21 ± 0.31		152	36.22 ± 0.51	

* *Probability of differences between the experimental and control means.*

Results

As can be readily appreciated from Table I, the amount of food eaten daily by the control mice increased with advancing pregnancy. It rose on gestation day 4 from the non-pregnancy level of 4.5 to 5 gm. per day to just over 5 gm., from which it began to climb on days 11 to 12, attaining its apex at over 6 gm. on day 16. After this there was a rapid decline, and the original level of 4.5 gm. per day was reached by day 18, the day preceding birth. The difference in mean food consumption between the first 10 and the last nine days of the pregnancy is statistically significant ($t = 5.21$, d.f. $= 559$, $P \ll .001$). Furthermore, no such fluctuation was found in four non-pregnant female mice observed (Table II), nor did these mice eat as much in 19 consecutive days as the pregnant mice did ($t = 5.40$ d.f. $= 635$, $P \ll .001$).

It is not surprising that the weight curves of the pregnant mice closely followed those of mean food consumption, and that the start of the ascent to the peak occurred at about days 10 to 11, since at approximately that time there is a burst of fetal growth (3). The dropoff in weight, of course, did not occur prior to parturition, as did that of food consumption. Several hours after parturition, which usually occurs during the early morning, the animals weighed a mean of 28.20 gm. (Table I).

What is the effect of cortisone on weight and food intake? Briefly, cortisone increased the amount of food eaten by, and decreased the weight of, pregnant female mice (Table I). Statistical tests of significance (Table I) indicate that neither of these tendencies was consistent for all gestation days on which animals were treated, but that, since the deviations on all days are in the same direction, the means for the entire treated periods compared with the control values for those times are undeniably different.

In addition, the influence of cortisone on weight and food consumption, as might have been anticipated, did not cease upon completion of the treatment. The effect on post-treatment food intake was reversed, however, and it fell below the control value (Table I). The post-treatment weights, on the other hand, continued to be the same as those of the treated animals and, of course, less than the control weights (Table I).

TABLE II

THE MEAN AMOUNT OF FOOD, IN GRAMS, EATEN BY FOUR NON-PREGNANT FEMALE MICE ON EACH OF 19 SUCCESSIVE DAYS

Day	Food	Day	Food
1	4.38	11	4.43
2	5.18	12	4.95
3	5.20	13	4.78
4	6.18	14	4.83
5	5.73	15	4.73
6	5.80	16	4.78
7	4.95	17	4.95
8	4.65	18	5.05
9	4.75	19	4.78
10	4.68		

Discussion

These results unequivocally indicate that the teratogenic properties of cortisone do not depend on a reduced food intake. Although Fraser and Fainstat (2) reported a reduction in the size of litters from cortisone-treated pregnant mice, this is not always so (6). Regardless of whether it is so or not, the differences in mean weight of the control and treated pregnant mice reported here cannot be due only to differences in number of fetuses, but are also a true reflection of differences in maternal body weights. This is indicated by the fact that the mean weights of control and treated females were very different even after parturition (Table I, day 19).

It has been noted frequently in the past that patients afflicted with any one of a large variety of pathological conditions, eat more upon treatment with cortisone than when not so treated (see van Putten *et al.* (7) for references). On the other hand, Borden *et al.* (1) have found that normal individuals, unlike the rheumatoid arthritics they studied, did not have increased appetites when given ACTH. The question arose, therefore, whether the improved appetite is merely a consequence of the euphoria that may be induced in ill persons by cortisone, or whether it might have a more direct and objective causation. To choose between these alternatives, van Putten *et al.* (7) measured the weights and food consumption of normal and burned rats treated with cortisone or ACTH. They concluded, when only insignificant differences were found, that the improved appetite of cortisone- and ACTH-treated patients "must be ascribed to a decrease of the sense of illness or to a general improvement in the patient's condition."

On the thesis that pregnancy causes a longer-lasting hyperactivity of the adrenal cortex than does a traumatic occurrence, such as burning, a comparison of the food consumption of control and cortisone-treated pregnant mice might be of some value in settling this question.

Winter *et al.* (8) found that, during treatment with 3 mgm. of cortisone given daily for six weeks, the growth of young adult rats was arrested. During this time, the absolute food intake, as compared with that of ad libitum fed controls, decreased; but the treated animals consumed 47% more food than control rats whose food intake was limited to restrict their weights to those of the treated animals. van Putten *et al.* (7) are content to cite this result as merely a "sharp decrease in food intake." Secondly, during a period of rapid accretion of weight, the cortisone-treated pregnant mice that are the subject of the present paper needed to eat more food than the control group to maintain a mean weight significantly below the control group's weight. Here, therefore, there was a large absolute and an immense relative increase in the food consumption of cortisone-treated animals.

Human beings have been the subjects of most of the investigations of this question, and because there is good reason to believe that no bodily activity of our species is entirely unaffected by the psyche, we must not doubt that cortisone has some psychological effect on appetite. Nevertheless, taking the

above facts into consideration, and not neglecting the well known catabolic action of cortisone on proteins, I propose the hypothesis that the relative and/or absolute increase in the food consumption of animals and diseased human beings treated with cortisone or ACTH is compensatory in nature, the treated individuals requiring larger amounts of food in the effort to overcome the metabolic consequences of large doses of these hormones.

Acknowledgment

I sincerely wish to thank Dr. F. Clarke Fraser, Department of Genetics, McGill University, Montreal, for a characteristically thorough and critical reading of this paper.

References

1. BORDEN, A. L., BRODIE, E. C., WALLRAFF, E. B., HOLBROOK, W. P., HILL, D. F., STEPHENS, C. A. L., JR., JOHNSON, R. B., and KEMMERER, A. R. J. Clin. Invest. 31 : 375. 1952.
2. FRASER, F. C. and FAINSTAT, T. D. Pediatrics, 8 : 527. 1951.
3. GRUNEBERG, H. The genetics of the mouse. Martinus Nijhoff, The Hague. 1952.
4. KALTER, H. Genetics, 39 : 185. 1954.
5. KALTER, H. Genetics, 39 : 975. 1954. (Abstr.).
6. KALTER, H. Unpublished data.
7. VAN PUTTEN, L. M., VAN BEKKUM, D. W., and QUERIDO, A. Acta Endocrinol. 12 : 159. 1953.
8. WINTER, C. A., SILBER, R. H., and STOERK, H. C. Endocrinology, 47 : 60. 1950.

ANTIOXIDANTS AS INHIBITORS OF LINOLEATE OXIDATION CATALYZED BY PLANT LIPOXIDASE AND BY HEMOLYZATES OF HUMAN ERYTHROCYTES[1]

By H. Bruce Collier and Sheila C. McRae

Abstract

Hemolyzates of human erythrocytes catalyzed the oxidation of linoleate at pH 7 but not at pH 9. Hence the erythrocytes contained no lipoxidase and the catalytic action was probably due to hemoglobin. However, the time–activity curves for hemolyzates and for crystalline hemoglobin were not identical in shape. The oxidation of linoleate at pH 7 by plant lipoxidase was powerfully inhibited by phenothiazine and by phenylhydrazine. These compounds, and also α-tocopherol and α-naphthol, inhibited the catalytic activity of hemolyzates and of crystalline hemoglobin. It is probable that phenothiazine and phenylhydrazine act as antioxidants in these systems. Antioxidants *in vivo* may possibly play a role in protecting the unsaturated fatty acids of the erythrocyte membrane from oxidation catalyzed by hemoglobin.

Introduction

Rose and György (15, 16) showed that tocopherol deficiency in rats caused the erythrocytes to become susceptible to hemolysis by dialuric acid. Inhibition of this hemolysis by α-tocopherol could be demonstrated both *in vivo* and *in vitro*. Gordon and de Metry (10) found that the erythrocytes of infants in certain abnormal states could be hemolyzed by hydrogen peroxide, and that administration of α-tocopherol to the infants restored the normal resistance to hemolysis.

Dam and his co-workers (1, 7) observed that phenothiazine and its derivatives afforded some protection against various symptoms of tocopherol deficiency in chicks and rats, and suggested (6) that these compounds might act as antioxidants. Dietary methylene blue was found to give partial protection against dialuric acid hemolysis of the erythrocytes of tocopherol-deficient rats (3). Moore, Sharman, and Ward (12) were unable, however, to confirm this effect of methylene blue. Collier and Dellert (4) showed that the addition of phenothiazine to the diet of tocopherol-deficient rats reduced the susceptibility of the red cells to dialuric acid, and also prevented the decrease in red-cell acetylcholinesterase activity that otherwise occurs.

As phenothiazine appeared to exhibit antioxidant activity *in vivo*, and as antioxidants are known to inhibit lipoxidase, it was decided to investigate the effect of phenothiazine and related compounds upon lipoxidase, and to determine whether this enzyme occurs in mammalian erythrocytes.

The lipoxidase of plant tissues is well known and its properties and mode of action have recently been reviewed by Holman (11) and by Tappel, Boyer, and Lundberg (22). Tappel has recently investigated the oxidation of

[1] *Manuscript received April 20, 1955.*
Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta., with financial assistance from the Defence Research Board of Canada, Grant No. DRB 9350-06.

unsaturated fatty acids by haem compounds (18, 21). He has also described (20) a method for the determination of lipoxidase activity in the presence of haem compounds, based on the fact that these compounds catalyze the oxidation of linoleate only when it is in the colloidal state (pH 7), while lipoxidase oxidizes linoleate in both the colloidal and homogeneous (pH 9) states. By this method Tappel found evidence for the absence of lipoxidase in animal tissues. Boyd and Adams (2) have recently used cyanide inhibition to distinguish haem catalysis from lipoxidase activity and have also failed to detect lipoxidase in animal tissues.

Methods

The substrate used was the highly purified linoleic acid of the Hormel Foundation. Crystalline hemoglobin was prepared from human blood by the method of Drabkin (8, 9). Purified plant lipoxidase was obtained from the Worthington Chemical Co. Alpha-tocopherol was the product of Nutritional Biochemicals Co. Phenylhydrazine hydrochloride was recrystallized from ethanol. Commercial phenothiazine (Merck) was recrystallized from benzene and the crystals were washed with hexane and dried *in vacuo*. This material was used to prepare phenothiazone (phenothiazine-5-oxide) by the method of Pummerer and Gassner (14); and the product was recrystallized from water.

The oxidation of linoleate by lipoxidase was followed by the manometric method of Tappel (18) in which the oxygen uptake at pH 7 is measured. Linoleic acid, 280 mgm., was treated with 1.00 ml. of 1 *N* ammonia, and water was added to a volume of 10 ml.; this resulted in a final concentration of 0.10 *M* ammonium linoleate. Summerson differential manometers were used, and into the main compartments of the right- and left-hand vessels were pipetted 0.60 ml. of the linoleate and 1.9 ml. of 0.10 *M* sodium phosphate buffer, pH 7.0. The enzyme solution, 0.5 ml., was placed in the side-arm of each right-hand flask, and an equal volume of buffer in the side-arm of each left-hand flask as a control. The vessels were gassed with oxygen for 10 min. and then the contents of the side-arms were tipped in. Manometric readings were taken every five minutes, for 30 min., at 25° C.

Compounds to be tested for inhibitory activity were added to the enzyme in the side-arm, and were therefore in contact with the enzyme during the 10 min. gassing period before addition of substrate. In the left-hand flask, inhibitor solution was replaced by an equal volume of water. As phenothiazine is insoluble in water, it was dissolved in ethanol and 0.1 ml. of the solution was added to the aqueous solution. An equal volume of ethanol was added to the control flasks. This concentration of ethanol had no effect on the enzyme activity, and in no case did the inhibitors alone appear to affect the rate of oxygen absorption.

To prepare substrate for the differentiation of haem catalysis from lipoxidase activity, the stock ammonium linoleate was diluted with 0.1 *M* ammonia – ammonium chloride buffer of pH 9.0 to a final concentration of 0.0009 *M* (20).

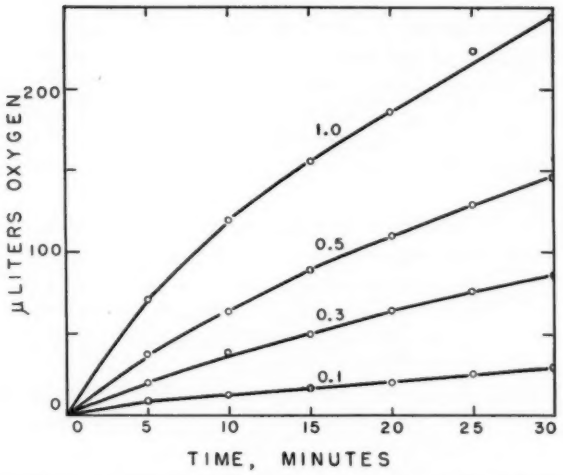


FIG. 1. Time–activity curves for oxidation of linoleate as catalyzed by plant lipoxidase at pH 7.0 and 25° C. Oxygen uptake in microliters is plotted against time in minutes. The concentration of enzyme, in mgm. per ml., is indicated for each curve.

Results

Inhibition of Plant Lipoxidase

The purified plant lipoxidase was used for a series of preliminary experiments. Time–activity curves were first obtained with lipoxidase concentrations in the range 0.1–1.0 mgm. per ml. of final mixture. Although oxygen uptake was not linear over the 30 min. period, the initial velocity varied directly with enzyme concentration, and relative enzyme activity was determined by estimating the initial velocity from the curves (Fig. 1).

The effect of various inhibitors upon the plant lipoxidase is summarized in Table I. The percentage inhibition was calculated from the decrease in initial velocity. It may be noted that phenothiazine and phenylhydrazine are powerful inhibitors of lipoxidase catalysis of linoleate oxidation.

TABLE I

EFFECT OF INHIBITORS UPON CATALYSIS OF LINOLEATE OXIDATION BY PLANT LIPOXIDASE

(0.15 mgm. of enzyme in 3 ml. total volume)

Inhibitor	Conc., *M*	Initial velocity, µl. O_2 per min.	Inhibition, %
Phenothiazine	0	9.9	0
	3.3×10^{-7}	9.9	0
	3.3×10^{-6}	7.2	27
	3.3×10^{-5}	5.9	41
	3.3×10^{-4}	2.7	79
Phenothiazone	0	5.6	0
	1.2×10^{-4}	4.8	14
Phenylhydrazine	0	6.0	0
	1.0×10^{-6}	4.1	22
	1.0×10^{-5}	0	100

Activity of Hemolyzates

Human erythrocytes from heparinized blood were washed three times in the centrifuge with isotonic saline; then 1 volume of packed cells was hemolyzed in 60 volumes of water. It was found that these hemolyzates catalyzed the oxidation of linoleate. As removal of the stromata in the high-speed centrifuge had little effect on the activity, whole hemolyzates were used in subsequent experiments.

The method of Tappel (20) was used to determine whether the oxidation was due to a true lipoxidase or to haem catalysis. Of the hemolyzate, 0.20 ml. was used per flask. No oxygen uptake was observed at pH 9.0 when the substrate was homogeneous ammonium linoleate. With colloidal substrate at pH 7.0 the initial rate of oxygen uptake was 6.4 microliters per minute, and the optimum activity was found to be at pH 7.0. Hence it was concluded that the catalysis of linoleate oxidation by hemolyzates was due to haem compounds, presumably the hemoglobin, and that no lipoxidase was present.

Time–activity curves obtained with hemolyzate showed a rapid initial oxygen uptake which was not directly proportional to enzyme concentration. Therefore a standard curve was constructed (Fig. 2) in which the oxygen uptake at the 10 min. period was plotted against the volume of hemolyzate employed. This was used to estimate the relative degree of catalytic activity in the presence of various inhibitors. The effect of inhibitors on catalysis by hemolyzate is recorded in Table II.

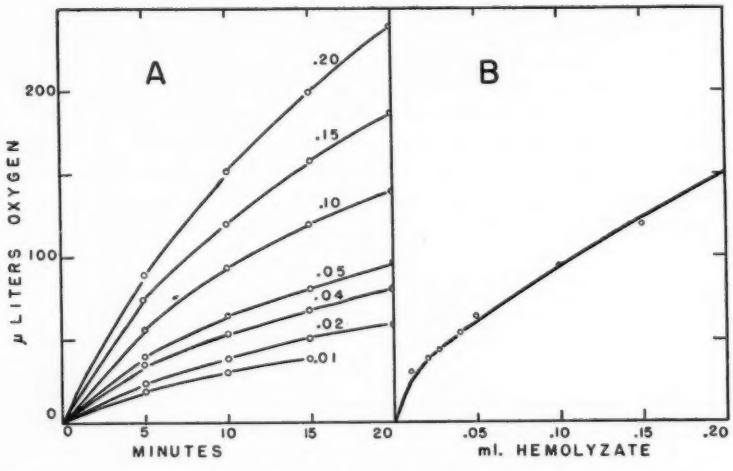


FIG. 2. Graph A represents time–activity curves for oxidation of linoleate by hemolyzates at pH 7.0 and 25° C. Oxygen uptake in microliters is plotted against time in minutes. The volume of 1 : 60 hemolyzate per 3.0 ml. reaction mixture is given for each curve.

Graph B is the standard curve for hemolyzates. The activity, expressed as microliters of oxygen absorbed in 10 min., is plotted against ml. of hemolyzate in 3.0 ml. total volume.

TABLE II

EFFECT OF INHIBITORS UPON CATALYSIS OF LINOLEATE OXIDATION BY HEMOLYZATE

(0.2 ml. of 1 : 60 hemolyzate in 3 ml. total volume)

% inhibition was estimated from the 10 min. readings and the standard curve

Inhibitor	Conc., *M*	Reaction velocity, μl. O_2 in 10 min.	Inhibition, %
α-Tocopherol	0	124	0
	3.3×10^{-5}	131	0
	3.3×10^{-4}	3	99
α-Naphthol	0	132	0
	3.3×10^{-5}	99	34
	3.3×10^{-4}	0	100
Phenylhydrazine	0	139	0
	1.0×10^{-5}	126	12
	1.0×10^{-4}	97	40
Phenothiazine	0	105	0
	3.3×10^{-7}	120	0
	3.3×10^{-6}	7	98
	3.3×10^{-5}	0	100
Phenothiazone	0	108	0
	1.5×10^{-5}	102	9
	1.5×10^{-4}	1	100

Activity of Hemoglobin and of Cytochrome-c

Crystalline hemoglobin was found to catalyze the oxidation of linoleate at an optimum pH of about 7.0. The time–activity curves were of the same shape as had been found for lipoxidase, and the initial velocity of oxygen uptake was proportional to hemoglobin concentration. In the presence of inhibitors, the percentage inhibition was estimated from the decrease in initial velocity. In the inhibition experiments, each flask contained about 1 mgm. of hemoglobin, which corresponds to 0.2 ml. of a 1 : 60 hemolyzate. The effect of various inhibitors on the catalytic action of hemoglobin is given in Table III.

TABLE III

EFFECT OF INHIBITORS UPON CATALYSIS OF LINOLEATE OXIDATION BY HEMOGLOBIN

(1 mgm. of Hb in 3 ml. total volume)

Inhibitor	Conc., *M*	Initial velocity, μl. O_2 per min.	Inhibition, %
α-Tocopherol	0	11.4	0
	3.3×10^{-5}	9.7	14
	3.3×10^{-4}	7.6	33
α-Naphthol	0	11.4	0
	3.3×10^{-5}	10.9	4
	3.3×10^{-4}	1.9	85
Phenothiazine	0	11.7	0
	3.3×10^{-5}	6.1	48
	3.3×10^{-4}	5.9	50
Phenothiazone	0	12.6	0
	1.0×10^{-5}	12.6	0
	1.0×10^{-4}	1.3	90

Cytochrome-*c* at a concentration of 0.3 mgm. per flask, also catalyzed the oxidation of linoleate. Phenothiazine at 3.3×10^{-4} *M* concentration brought about almost complete inhibition.

Discussion

Phenothiazine and phenylhydrazine were observed to be powerful inhibitors of plant lipoxidase. Alpha-tocopherol was not tested, as it had already been shown by Tappel *et al.* (22) to be an inhibitor of crude soybean lipoxidase, as were hydroquinone, propyl gallate, and nordihydroguaiaretic acid.

The catalytic activity of erythrocyte hemolyzates was apparently due to the hemoglobin as there was no evidence of the presence of lipoxidase. Nevertheless, the time–activity curves obtained with hemolyzates differed from those obtained with hemoglobin or with lipoxidase, so that some factors in addition to the hemoglobin must have influenced the reaction. Phenothiazine, phenothiazone, α-tocopherol, and α-naphthol were powerful inhibitors of hemolyzates. The catalytic activity of crystalline hemoglobin was inhibited by phenothiazine, α-tocopherol, and α-naphthol. Tappel (21) had found that the catalytic action of hemoglobin was inhibited by phenolic antioxidants, including α-tocopherol. He noted that these compounds increased the induction period of oxygen uptake, but we did not observe this phenomenon in our experiments.

Tappel (21) also observed that methylene blue, a phenothiazine derivative, inhibited haem catalysis of unsaturated fatty acids, but stimulated the *autoxidation* of oleic acid. He suggested that the basic thiazines may form hemochromogens with the iron of haem compounds, while α-tocopherol may destroy the fat peroxides, and thus prevent the initiation of haem-catalyzed free-radical chain reaction oxidation.

Tappel (19) has proposed that α-tocopherol may play a role, *in vivo*, in preventing the oxidation of unsaturated fatty acids by haem compounds. Destruction of the essential unsaturated fatty acids by haem catalysis would presumably result in symptoms of essential fatty acid deficiency. It is not clear whether α-tocopherol may play such a role in the blood. The high concentration of hemoglobin in the erythrocyte, together with oxygen, might cause the oxidation of unsaturated fatty acids in the cell membrane.* Thus, there is a possibility that α-tocopherol might play a role in protecting components of the erythrocyte membrane from oxidation catalyzed by hemoglobin. Such a possibility deserves further investigation.

It is of interest that phenothiazine, in our experiments, exhibits antioxidant activity. Murphy, Ravner, and Smith (13) reported the compound to be a powerful antioxidant when used in lubricating oils. Although phenothiazine can protect the erythrocytes against damage from α-tocopherol deficiency (4), it is also hemolytic when fed to animals in large amounts (5, 17). Methylene blue and phenylhydrazine are also substances that cause hemolytic anemia,

* *Unfortunately there is virtually no information concerning the nature of the unsaturated fatty acids in the lipids of the erythrocyte stroma.*

but can act as antioxidants as well. The explanation may lie in the fact, pointed out by Dam (6), that an antioxidant may give its optimum effect at a certain concentration, while an increase above this concentration may result in a pro-oxidant effect.

References

1. AAES-JØRGENSEN, E., DAM, H., and GRANADOS, H. Acta Pharmacol. Toxicol. 7 : 171. 1951.
2. BOYD, D. H. J. and ADAMS, G. A. Can. J. Biochem. Physiol. 33 : 191. 1955.
3. CHRISTENSEN, F. and DAM, H. Acta Pharmacol. Toxicol. 7 : 167. 1951.
4. COLLIER, H. B. and DELLERT, E. E. Proc. XIX Intern. Physiol. Cong. 274. 1953.
5. COLLIER, H. B. and MACK, G. E., JR. Can. J. Research, E, 22 : 1. 1944.
6. DAM, H. Experientia, Suppl. I : 195. 1953.
7. DAM, H., KRUSE, I., PRANGE, I., and SØNDERGAARD, E. Acta Physiol. Scand. 22 : 299. 1951.
8. DRABKIN, D. L. J. Biol. Chem. 164 : 703. 1946.
9. DRABKIN, D. L. Arch. Biochem. 21 : 224. 1949.
10. GORDON, H. H. and DE METRY, J. P. Proc. Soc. Exptl. Biol. Med. 79 : 446. 1952.
11. HOLMAN, R. T. Trans. Am. Assoc. Cereal Chemists, 11 : 135. 1953.
12. MOORE, T., SHARMAN, I. M., and WARD, R. J. Biochem. J. 53 : xxxi. 1953.
13. MURPHY, C. M., RAVNER, H., and SMITH, N. L. Ind. Eng. Chem. 42 : 2479. 1950.
14. PUMMERER, R. and GASSNER, S. Ber. 46 : 2310. 1913.
15. ROSE, C. S. and GYÖRGY, P. Blood, 5 : 1062. 1950.
16. ROSE, C. S. and GYÖRGY, P. Am. J. Physiol. 168 : 414. 1952.
17. SWALES, W. E., COLLIER, H. B., and ALLEN, D. Can. J. Research, D, 20 : 349. 1942.
18. TAPPEL, A. L. Arch. Biochem. and Biophys. 44 : 378. 1953.
19. TAPPEL, A. L. Arch. Biochem. and Biophys. 47 : 223. 1953.
20. TAPPEL, A. L. Food Research, 18 : 104. 1953.
21. TAPPEL, A. L. Arch. Biochem. and Biophys. 50 : 473. 1954.
22. TAPPEL, A. L., BOYER, P. D., and LUNDBERG, W. O. J. Biol. Chem. 199 : 267. 1952.

FACTORS AFFECTING THE ACTIVITY OF ALLOXAN *IN VITRO*[1]

By S. J. Klebanoff[2]

Abstract

The effect of alloxan on the oxygen uptake and the succinic dehydrogenase activity of rat liver homogenates has been studied in an attempt to gain some insight into the activity of alloxan within the cell. The increased oxygen uptake on the addition of alloxan to liver homogenates was found to be non-enzymatic in nature and to depend on some heat stable factor or factors in the preparation. The ability of reduced glutathione to substitute for the homogenate suggests that this substance may be concerned. It is thought that alloxan is reduced to dialuric acid by a number of factors in the homogenate, and that the increased oxygen uptake results from the spontaneous reoxidation of this latter substance. Experiments with dialuric acid tend to confirm this hypothesis. The inactivation of succinic dehydrogenase by alloxan is greatly influenced by the pH of the solution in which the alloxan is dissolved, as well as by the pH of the enzyme preparation, with acidity increasing and alkalinity decreasing the effect. The progressive nature of the inactivation with time is shown to depend upon the lowering of the pH which is brought about by the addition of alloxan to an unbuffered enzyme mixture. The experimental results are discussed in relation to the possible importance of pH in the selective toxicity of alloxan.

Introduction

One of the most interesting problems arising from a consideration of the mechanism of action of alloxan is the selective nature of the toxicity. It is generally accepted that the destructive action of alloxan is a result of a chemical interaction between alloxan and some essential component of the cell. Alloxan is an exceedingly strong oxidizing agent with a special affinity for sulphydryl groups. It is well known that many enzymes and coenzymes have free suplhydryl groups which are essential for their activity. It was therefore suggested by Lazarow (16, 17) that the toxicity of alloxan may be due to the inactivation of these essential sulphydryl compounds within the cell. However, despite the very general distribution of sulphydryl compounds throughout all the cells of the body, the administration of diabetogenic doses of alloxan leads to a relatively specific destruction of the beta cells of the islets of Langerhans.

A consideration of the factors affecting the rate of degradation of alloxan within the body offers a possible explanation for this selectivity (15, 17). It has been repeatedly emphasized that the spontaneous conversion of alloxan to alloxanic acid is extremely sensitive to small changes in pH within the physiological pH range, with acidity decreasing and alkalinity increasing the reaction rate (15, 20, 27). It has therefore been suggested that the selective action of alloxan may be due, in part, to the beta cells having a

[1] *Manuscript received April 26, 1955.*

Contribution from the Department of Biochemistry, University College, London, and the Department of Pathological Chemistry, University of Toronto, Toronto, Ontario.

[2] *Medical Research Fellow, National Research Council, Canada. Present address: Department of Pathological Chemistry, University of Toronto, Toronto, Ontario.*

more acid pH than the body tissues in general. If this were so, the inactivation of alloxan within these cells would be relatively slow, and its toxic action correspondingly prolonged (14, 15).

The effect of alloxan on rat liver homogenates has been investigated in an attempt to gain some insight into the behavior of alloxan within the cell, with particular reference to the importance of pH in this activity.

Materials and Methods

Rat liver homogenates were prepared in 0.25 *M* sucrose by the method of Potter and Elvehjem (22) to give a tissue concentration of 1 in 10. The animals used were well fed male rats of an inbred albino strain, aged about 10 weeks, and weighing between 200 and 250 gm. Alloxan (B.D.H.) and dialuric acid (Lights) were freshly dissolved in *N*/100 hydrochloric acid. Thus prepared, alloxan remained stable for many days. Dialuric acid, however, was unstable, as evidenced by the oxygen uptake of the solution, and so had to be prepared immediately prior to use. The reduced glutathione was kindly supplied by the Distillers Co. (Biochemicals) Ltd. The oxidized glutathione was prepared by iodine oxidation (23).

The oxygen uptake was determined in an atmosphere of air at 37° C. using a Warburg respirometer. The contents of the flasks are as detailed below.

The succinic dehydrogenase activity was estimated using a modified Thunberg methylene blue technique (5). Rat liver homogenate (1.0 cc.), 1.0 cc. of 0.067 *M* phosphate buffer at various pH levels, 0.7 cc. of distilled water, and 0.3 cc. of 0.025 *M* alloxan were placed in the main tube, while 0.1 cc. of a 1 : 1000 solution of methylene blue and 0.2 cc. of a 0.2 *M* solution of sodium succinate were placed in the hollow stopper. The tube was evacuated and following an incubation period, unless otherwise stated, of 10 min., the contents of the stopper were tipped into the main tube. The time taken for the complete reduction of methylene blue at 37° C. was then determined. The sodium succinate was added to the hollow stopper rather than to the main tube, since it has been demonstrated that the inactivation of succinic dehydrogenase by alloxan is inhibited by the presence of succinate (12).

Results

The Effect of Alloxan on the Oxygen Uptake of Rat Liver Homogenates

Bernheim has reported that the addition of alloxan to various tissue preparations resulted in a marked increase in the oxygen uptake (3). A confirmation of this observation is found in Fig. 1. A rat liver homogenate was allowed to respire in the absence of alloxan for 30 min. The addition of *N*/100 hydrochloric acid at this point had no effect on the oxygen uptake, whereas alloxan at a final concentration of 0.00083 *M* caused a rapid acceleration. The increase in oxygen uptake was most pronounced during the initial five minute period, and then gradually declined.

Various possible explanations for this phenomenon have been suggested. Bernheim (3) was originally led to this study by the close structural relationship between alloxan and the alloxazine moiety of the flavin nucleotides. Dixon and Zerfas (7) subsequently demonstrated that alloxan can act as a hydrogen acceptor in the enzymatic dehydrogenation of various tissue metabolites, by virtue of the oxidation–reduction reaction alloxan to dialuric acid, and they suggested that this might explain the findings of Bernheim. If the acceleration of the oxygen uptake is due to the ability of alloxan to act as a hydrogen acceptor in biological systems, it must depend, in the first instance, on the enzymatic dehydrogenation of a tissue metabolite. Bernheim had demonstrated that a stimulation occurs in the absence of an added metabolite, an observation which has been confirmed here (Fig. 1). In the experiment described below, evidence is presented which indicates that the increased oxygen uptake observed following the addition of alloxan does not depend on the enzymatic oxidation of an endogenous metabolite, and that in fact, it can be elicited in the complete absence of an active enzyme system.

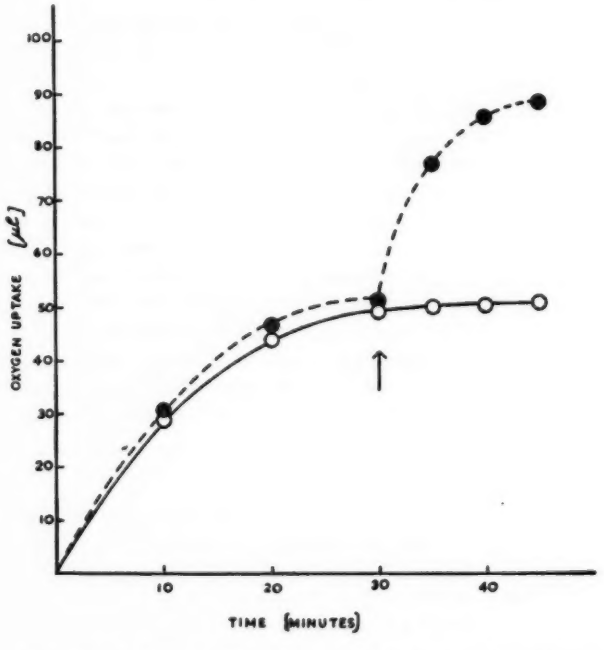


FIG. 1. The effect of alloxan on the oxygen uptake of rat liver homogenates. Flasks contained 1.0 cc. of 0.067 *M* phosphate buffer pH 6.0, 1.0 cc. of the homogenate, and 0.8 cc. distilled water. The side arm contained 0.2 cc. *N*/100 HCl (——○——) or 0.0125 *M* alloxan monohydrate(— — ● — —), which was added to main compartment at arrow.

TABLE I

THE ROLE OF AN ACTIVE ENZYME SYSTEM IN THE STIMULATION OF THE OXYGEN UPTAKE BY ALLOXAN

	Flask number							
	1	2	3	4	5	6	7	8
Experimental procedure								
0.0125 *M* alloxan in *N*/100 HCl (side arm)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
0.067 *M* phosphate buffer, pH 6.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
0.1 *M* arsenite			0.2			0.2		
0.2 *M* sodium fluoride		0.2			0.2			
Distilled water	0.8	0.6	0.6	0.8	0.6	0.6	0.8	1.8
Homogenate from well fed animal	1.0	1.0	1.0					
Homogenate from starved animal (72 hr.)				1.0	1.0	1.0		
Boiled homogenate from well fed animal							1.0	
Results								
O_2 uptake (μl.) during 5 min. preceding alloxan	5	4	3	6	2	2	3	0
O_2 uptake (μl.) during 5 min. following alloxan	26	22	20	34	18	15	36	0

Warburg flasks were set up as shown in Table I. Following a 30 min. incubation period, the alloxan present in the side arm was tipped into the main compartment. The oxygen uptake during the five minutes preceding and the five minutes following the addition of alloxan was recorded.

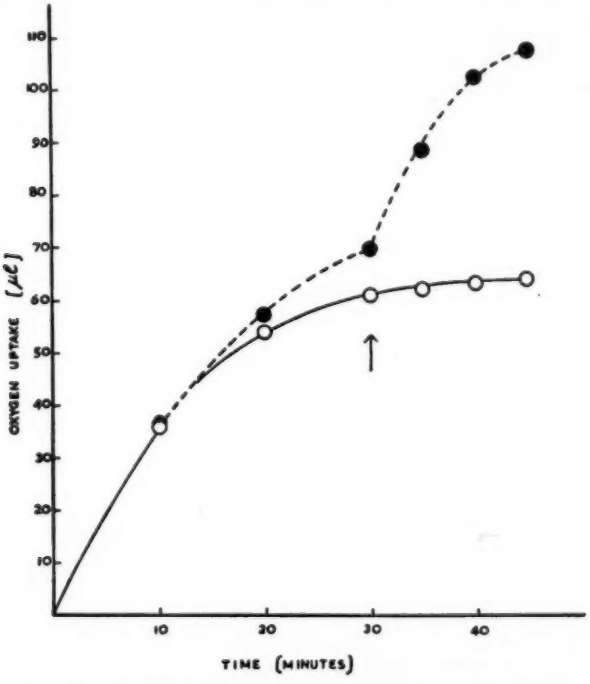


FIG. 2. The effect of dialuric acid on the oxygen uptake of rat liver homogenates. As in Fig. 1, with the substitution of 0.0125 *M* dialuric acid for alloxan. ——○—— HCl; — — ● — — dialuric acid.

The results are shown in Table I. The addition of alloxan, at a final concentration of 0.00083 *M* to an untreated homogenate, prepared from a normally fed animal (Flask 1) resulted in an increase in the oxygen uptake from 5 to 26 μl. The stimulatory effect of the alloxan was not diminished by the use of an homogenate prepared from an animal which had been starved for 72 hr. to lower the concentration of endogenous substrates (Flask 4). Indeed, the oxygen uptake was higher (34 μl.) than that of an homogenate prepared from a normally fed animal (26 μl.). The addition of arsenite or fluoride to homogenates prepared from either a well fed or starved animal, in order to inhibit endogenous enzymatic oxidations, did not prevent the increase in the oxygen uptake on the addition of alloxan (Flasks 2, 3, 5, and 6), although the absolute level of the oxygen absorbed by the arsenite or fluoride-treated homogenates was lower than that of the untreated preparations. Of particular interest was the demonstration that the acceleration of the oxygen uptake was greatest (3 to 36 μl.) when the enzymes of the homogenate were completely destroyed by boiling (Flask 7). Since no uptake of oxygen occurred in the absence of an homogenate (Flask 8), the stimulation of the oxygen uptake by alloxan would appear to be non-enzymatic in nature, and dependent on some heat stable factor or factors present in the homogenate.

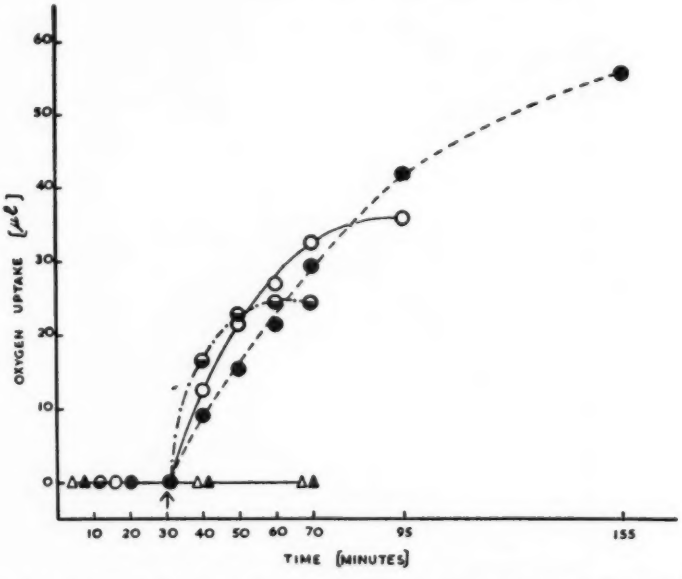


FIG. 3. The effect of alloxan on the oxygen uptake in the presence of glutathione. 0.2 cc. of 0.0125 *M* alloxan monohydrate in side arm was added (at arrow) to main compartment, which contained 1.0 cc. of 0.067 *M* phosphate buffer pH 6.0, distilled water to 3.0 cc., and ——▲—— no glutathione; —·—●—·— 0.1 cc; ——○—— 0.2 cc; — —●— — 0.4 cc. of 0.25 *M* reduced glutathione; and ——△—— 0.4 cc. of 0.25 *M* oxidized glutathione.

Dialuric acid, a simple reduction product of alloxan, is readily oxidized non-enzymatically by atmospheric oxygen to form alloxan (10, 24). As can be seen in Fig. 2, the addition of dialuric acid to a rat liver homogenate resulted in a substantial increase in the oxygen uptake, which followed a pattern similar to that obtained on the addition of alloxan (Fig. 1). This suggests that the stimulation of the oxygen uptake by alloxan may be due to the reoxidation of dialuric acid formed on the reduction of alloxan by various components of the homogenate.

The results, shown in Fig. 3, suggest that reduced glutathione may be one of the factors present in the homogenate which is required for the stimulation of the oxygen uptake by alloxan. Alloxan at a final concentration of 0.00083 *M* was added to a solution of glutathione in phosphate buffer at pH 6.0 in the absence of an homogenate. In the absence also of glutathione, or in the presence of oxidized glutathione, the addition of alloxan did not result in an oxygen uptake. However in the presence of reduced glutathione, there was a substantial oxygen uptake which increased as the glutathione concentration was increased. Similarly, the addition of dialuric acid to a solution containing reduced glutathione in the absence of an homogenate caused an increase in the oxygen uptake in a manner identical to that noted on the addition of alloxan to similar systems (Fig. 4). However, unlike alloxan, the presence of dialuric acid in the side arm for 30 min. prior to its addition to the main compartment was associated with a distinct oxygen uptake as a result, presumably, of the spontaneous oxidation to alloxan.

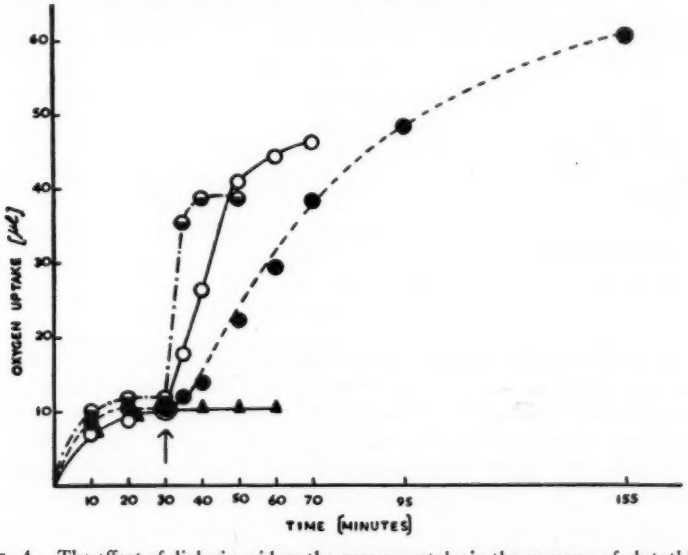


FIG. 4. The effect of dialuric acid on the oxygen uptake in the presence of glutathione. As in Fig. 3, with the substitution of 0.0125 *M* dialuric acid for alloxan. ——▲—— no glutathione, —·—●—·— 0.1 cc., ——○—— 0.2 cc., — —●— — 0.4 cc. reduced glutathione.

The Inactivation of Succinic Dehydrogenase by Alloxan

Hopkins, Morgan, and Lutwak-Mann (12) have demonstrated the inactivation of succinic dehydrogenase by alloxan *in vitro*. This observation has assumed added importance with the advent of the discovery of alloxan diabetes since Lazarow (16) has suggested that the inactivation of essential sulphydryl enzymes may be the mechanism by which alloxan exerts its diabetogenic effect. The inactivation of succinic dehydrogenase by alloxan has therefore been reinvestigated with particular reference to the role of pH in this reaction.

Test tubes containing alloxan and phosphate buffer at various pH levels were preincubated for one hour at 37° C., in an atmosphere of air. An aliquot of the alloxan solution sufficient to produce a final concentration of 0.0025 *M* was then added to the rat liver homogenate and the succinic dehydrogenase activity was estimated. The results, as given in Table II, show that if the alloxan, prior to its addition to the enzyme mixture, is incubated in an acid buffer mixture, it exerts, when added to the enzyme system, a powerful inhibitory effect on the succinic dehydrogenase activity. However, when the preincubation of the alloxan is carried out at an alkaline pH, the alloxan is seen to lose completely its ability to inactivate the enzyme.

TABLE II

THE EFFECT OF THE PREINCUBATION OF ALLOXAN AT VARIOUS pH LEVELS ON THE INACTIVATION OF SUCCINIC DEHYDROGENASE BY ALLOXAN

	Tube No.								
	1	2	3	4	5	6	7	8	9
Preincubation of alloxan									
0.067 *M* phosphate buffer									
pH	6.0		6.0	6.4	6.8	7.0	7.2	7.4	7.8
Vol.	0.5		0.5	0.5	0.5	0.5	0.5	0.5	0.5
0.05 *M* alloxan in *N*/100 HCl		0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
N/100 HCl	0.5	0.5							
Succinic dehydrogenase activity									
Methylene blue reduction time, min. (and sec.)	2^{10}	40^{00}	43	28	13	8	4^{15}	2^{10}	2^{10}

In the foregoing experiment, alloxan, preincubated in buffer at various pH levels, was added to an enzyme preparation maintained at a pH of 6.0. In the following experiment, alloxan dissolved in *N*/100 hydrochloric acid, and therefore at a constant pH, was added to an enzyme preparation which was maintained at various pH levels (6.0, 6.6, 7.0, 7.4, and 8.0) with phosphate buffer. The succinic dehydrogenase activity was determined and compared to a control value, obtained by the addition of *N*/100 hydrochloric acid to the enzyme system at each pH level. The concentration of alloxan was then altered until the reduction time was increased to twice the control value for that particular pH.

The results, as shown in Table III, indicate that the concentration of alloxan required to double the reduction time following a 10 min. incubation period was substantially increased as the pH of the enzyme preparation was raised from 6.0 to 8.0. Thus, at pH 8.0, approximately six times the amount of alloxan is required as is needed at pH 6.0, whereas the amount required at pH 7.0 is almost twice that needed at pH 6.6.

TABLE III

THE EFFECT OF THE pH OF THE ENZYME SYSTEM ON THE INACTIVATION OF SUCCINIC DEHYDROGENASE BY ALLOXAN

pH	Alloxan concentration
8.0	*M*/500
7.4	*M*/850
7.0	*M*/1100
6.6	*M*/2000
6.0	*M*/3100

The demonstration that the inactivation of succinic dehydrogenase by alloxan, presumably at neutral pH, increased with time over a 30 min. period (12) is difficult to reconcile with the extreme lability of alloxan at this pH. An attempt was therefore made to repeat this observation. Into each of four centrifuge tubes were placed 2.2 cc. of the homogenate, 1.4 cc. of distilled water, and 0.4 cc. of alloxan dissolved in *N*/100 hydrochloric acid, to make a final concentration of 0.02 *M*. The tubes were incubated in an atmosphere of air at 37° C. for periods varying from one minute and 30 sec., to 30 min. They were then centrifuged for five minutes at 900 × *g*, the supernatant discarded, and the residue washed with 2.0 cc. of 0.25 *M* sucrose. An aliquot was taken for the determination of succinic dehydrogenase activity. A control tube, containing no alloxan, was treated as above, following an incubation period of 30 min.

The results are shown in Table IV. The incubation of alloxan at a concentration of 0.02 *M* with the enzyme preparation for one minute and 30 sec.,

TABLE IV

THE EFFECT OF THE LENGTH OF INCUBATION ON THE INACTIVATION OF SUCCINIC DEHYDROGENASE BY ALLOXAN IN AN UNBUFFERED SYSTEM

Alloxan concentration	Preincubation time, min. (and sec.)	Methylene blue reduction time, min.
None	—	10
M/50	1[30]	18
M/50	5	25
M/50	15	53
M/50	30	> 200

increased the reduction time from 10 min. to 18 min. As the length of the incubation period was increased, the reduction time was further lengthened, until, following an incubation of 30 min., the methylene blue was not completely reduced at the end of 200 min. These results are in accord with Hopkins *et al.* (12) and indicate that, under the conditions employed, the inactivation of succinic dehydrogenase by alloxan is continuous in nature over a 30 min. period.

However, in the experiment just described, and in that of Hopkins *et al.* (12), alloxan was added to a tissue preparation which was not externally buffered. Since the degradation of alloxan results in the production of the strongly acidic alloxanic acid, the effect of alloxan on the pH of the unbuffered homogenate was investigated with the use of indicators. When added to the homogenate as in the previous experiment, alloxan resulted in a fall in pH from 7.2 to 5.2 within the first minute of incubation, the pH remaining constant at 5.2 over a 30 min. period. An equivalent volume of N/100 hydrochloric acid caused a fall in pH from 7.2 to 6.7.

In the following experiment an attempt is made to prevent the fall in pH consequent on the addition of alloxan, by fortifying the homogenate with buffer at pH 7.4. The experiment was performed as described above except that the centrifuge tube contained 0.067 *M* phosphate buffer at pH 7.4 instead of water. The addition of alloxan to this system resulted in a fall in pH from 7.4 to 6.9. The results as shown in Table V indicate that the inactivation of succinic dehydrogenase by alloxan in a system in which the pH is stabilized by an external buffer at 6.9 does not increase with time over a 30 min. period. The inactivation proceeds at a substantial rate for the first five minutes, with only a slight increase in the inactivation resulting from further incubation. It is of interest further that the inactivation is much less complete in the buffered system than it is in the unbuffered system. The reduction time was 19 min. following a 30 min. incubation period, at pH 6.9, as compared to over 200 min. following a similar incubation period at pH 5.2. Thus the progressive inactivation would appear to depend on the fall in pH consequent on the addition of alloxan to an unbuffered homogenate.

TABLE V

THE EFFECT OF THE LENGTH OF INCUBATION ON THE INACTIVATION OF SUCCINIC DEHYDROGENASE BY ALLOXAN IN A BUFFERED SYSTEM

Alloxan concentration	Preincubation time, min. (and sec.)	Methylene blue reduction time, min. (and sec.)
None	—	6
M/50	1[30]	9
M/50	5	16[30]
M/50	10	18
M/50	15	19
M/50	30	19

Discussion

Because of the destructive action of the blood and intercellular fluids, only a portion of the administered alloxan reaches the cells in an intact state. It would seem highly probable that alloxan must pass into the cell before its toxic action is effected. Although there is abundant pharmacological evidence which emphasizes the importance of membrane permeability in the action of toxic substances, in the present state of our knowledge, the importance of this factor in the toxicity of alloxan can only be conjectured. One point, however, is worth mentioning. Hober (11) has summarized the evidence in support of the generalization that neutral molecules pass through cell membranes more readily than do their ionized forms. If this generalization can be applied to alloxan; an interesting relationship between membrane permeability, and pH, may be operative. Since alloxan is a weak acid, with a pK of approximately 7.0 (20, 25), a slight fall in pH in the physiological range will result in quite a substantial increase in the concentration of the neutral molecule. If a corresponding increase in the passage of alloxan into the cell occurs, then local variations in pH may lead to a selective accumulation within certain cells of the body.

Within the cell, alloxan immediately comes under the influence of a number of conditioning factors. The action of alloxan on tissue preparations has been studied in an attempt to clarify some of these factors, although it is evident that results obtained *in vitro* must be interpreted with caution with respect to activity *in vivo*.

Bernheim has demonstrated that alloxan causes an acceleration of the oxygen uptake of tissue preparations (3), an observation which has been confirmed here (Fig. 1). The experiments presented above suggest that the oxygen uptake results from the non-enzymatic reoxidation of dialuric acid. Alloxan when added to an homogenate, and presumably also within the cell, enters into a readily reversible oxidation–reduction system—alloxan to dialuric acid—which transfers hydrogens from many sources to molecular oxygen.

The results shown in Fig. 3 suggest that glutathione may be one of the factors present in the homogenate which causes the reduction of alloxan. It is not suggested, however, that glutathione is the only factor or even necessarily the most important factor in the homogenate which is capable of this reaction. Siliprandi (28) has demonstrated an oxygen uptake on the addition of alloxan to a solution containing cysteine. Alloxan can also be reduced by other sulphydryl compounds (18, 12), alpha amino compounds (29), reduced coenzyme I (6), ascorbic acid (26), and a number of substrates undergoing biological dehydrogenation (7). Indeed, that this latter reaction is operative is suggested by the depression of the stimulation of the oxygen uptake in the presence of fluoride or arsenite (Table I). The high activity of the boiled preparation is probably a result of the denaturation of the proteins, a process which releases masked sulphydryl groups (1). It is of interest that the stimulatory effect of alloxan is greater when an homogenate

prepared from a starved animal is used, as compared to one from a well fed animal (Table I). This may be related to the demonstration that the inactivation of succinic dehydrogenase (and presumably other sulphydryl enzymes) is inhibited by the presence of its substrate (12). This is of particular interest as a possible explanation, in part, for the potentiating effect of starvation (13) or the protective role of hexoses (4, 2) in alloxan diabetes.

The oxygen uptake, therefore, is taken as evidence for the operation of the oxidation–reduction system, alloxan to dialuric acid. The conversion of alloxan to alloxanic acid appears to be a major pathway for the degradation of alloxan in the body (27). Since this reaction is markedly pH dependent, with acidity decreasing, and alkalinity increasing the reaction rate, the level of the alloxan–dialuric acid system will be controlled to a large extent by the pH of the medium. That the toxicity depends on the level of the oxidation–reduction system, and is therefore also pH dependent, is suggested by the relationship of pH to the inactivation of succinic dehydrogenase by alloxan. The amount of alloxan required to produce an equivalent degree of inactivation in unit time (i.e. double the methylene blue reduction time in 10 min.) becomes progressively lower as the pH of the enzyme system is decreased (Table III). Furthermore, at the higher pH levels the inactivation proceeds for a short period only (Table V) whereas at the lower pH levels, the inactivation becomes progressively greater with time (Table IV).

It is a widely held view that alloxan acts only during the first 5 to 10 min. following its injection into the body. This concept has developed from the demonstration that alloxan is present in blood for this short period only (9, 19). However, if the information gained from *in vitro* experiments can be applied to the behavior of alloxan *in vivo*, then the short life of alloxan within the cell would lead to only a slight inactivation of succinic dehydrogenase. If this can be applied to other essential substances as well, it would appear highly unlikely that this partial inactivation would result in the extensive cellular damage found. However, if the intercellular pH is at a low level, then the action of alloxan will be prolonged and the inactivation progressively increased.

It should be borne in mind that the fall in pH, consequent to the conversion of alloxan to alloxanic acid, may affect the activity of the tissue preparation, quite apart from its stabilizing influence on alloxan. Thus Ecker *et al.* (8) found that the inhibition by alloxan of the complement activity of rabbit blood *in vitro* was associated with a fall in pH to 6.0 or below, and that the activity was completely restored by neutralization to pH 7.2. It is not known to what extent alloxan lowers the pH of different cells. However, it is of interest that a second diabetogenic substance, dehydroascorbic acid, is a neutral molecule, which is converted to a strongly acidic substance, ascorbic acid, *in vivo* (21).

It has been suggested that during the secretion of an alkaline pancreatic juice, an acid tide is produced which may be sufficient to maintain the islet cells in a state of relative acidity (14, 15). As a result, the inactivation of alloxan within these cells would be relatively slow, and its toxic action,

correspondingly prolonged. Furthermore, a state of relative acidity may lead to a selective accumulation of alloxan within these cells. The unique anatomical arrangement of the mammalian pancreatic cells would appear to be ideal for the direct diffusion of acid from the electrolyte secreting cells of the pancreas to the islet tissue. However, it should be emphasized that the alpha cells are relatively resistant to alloxan, despite an anatomical arrangement similar to the more susceptible beta cells. This selectivity within the islets of Langerhans can not be adequately explained by the foregoing hypothesis.

The selective nature of the diabetogenic action of alloxan is an exceedingly complex problem and it is not suggested that the foregoing hypothesis is sufficient to account for the many and varied observations regarding this toxicity. However, it is meant to emphasize that a functional relationship may exist between the exocrine and endocrine portion of the pancreas which corresponds with the extremely close anatomical relationship between these two tissues.

Acknowledgments

I would like to express my gratitude to Dr. A. L. Greenbaum for his encouragement and advice throughout the course of this investigation, and to Professor J. A. Dauphinee for his assistance in the preparation of the manuscript.

References

1. Anson, M. L. Advances in Protein Chem. 2 : 361. 1945.
2. Arteta, J. L., Konig, G., and Carballido, A. J. Endocrinology, 10 : 342. 1954.
3. Bernheim, F. J. Biol. Chem. 123 : 741. 1938.
4. Bhattacharya, G. Science, 117 : 230. 1953.
5. Burris, R. H. Manometric techniques and tissue metabolism. 2nd ed. *Edited by* Umbreit, W. W., Burris, R. H., and Stauffer, J. F. Burgess Publishing Company, Minneapolis. 1949. p. 105.
6. Cooperstein, S. J., Lazarow, A., and Patterson, J. W. Unpublished data. *See* A. Lazarow (17).
7. Dixon, M. and Zerfas, L. G. Biochem. J. 34 : 371. 1940.
8. Ecker, E. E., Kalina, R., and Pillemer, L. Enzymologia, 7 : 307. 1938.
9. Gomori, G. and Goldner, M. G. Proc. Soc. Exptl. Biol. Med. 58 : 232. 1945.
10. Hill, E. S. J. Biol. Chem. 85 : 713. 1930.
11. Hober, R. Physical chemistry of cells and tissues. J. & A. Churchill, Ltd., London. 1947. p. 259.
12. Hopkins, F. G., Morgan, E. J., and Lutwak-Mann, C. Biochem. J. 32 : 1829. 1938.
13. Kass, E. H. and Waisbren, B. A. Proc. Soc. Exptl. Biol. Med. 60 : 303. 1945.
14. Klebanoff, S. J. and Greenbaum, A. L. J. Endocrinol. 10 : xix. 1954.
15. Klebanoff, S. J. and Greenbaum, A. L. J. Endocrinol. 11 : 314. 1954.
16. Lazarow, A. Physiol. Revs. 29 : 49. 1949.
17. Lazarow, A. Experimental diabetes and its relation to the clinical disease. Blackwell Scientific Publications, Oxford. 1954. p. 49.
18. Lazarow, A. and Cooperstein, S. J. Abstr. 19th intern. physiol. Congr. Montreal, 547. 1953.
19. Leech, R. S. and Bailey, C. C. J. Biol. Chem. 157 : 525. 1945.
20. Patterson, J. W., Lazarow, A., and Levey, S. J. Biol. Chem. 177 : 187. 1949.
21. Patterson, J. W. and Mastin, D. W. Am. J. Physiol. 167 : 119. 1951.
22. Potter, V. R. and Elvehjem, C. A. J. Biol. Chem. 114 : 495. 1936.
23. Rall, T. W. and Lehninger, A. L. J. Biol. Chem. 194 : 119. 1952.
24. Richardson, G. M. Biochem. J. 26 : 1959. 1932.
25. Richardson, G. M. and Cannan, R. K. Biochem. J. 23 : 68. 1929.
26. Ruben, J. A. and Tipson, R. S. Science, 101 : 536. 1945.
27. Seligson, D. and Seligson, H. J. Biol. Chem. 190 : 647. 1951.
28. Siliprandi, N. Boll. soc. ital. biol. sper. 25 : 638. 1949.
29. Strecker, A. Ann. Chem. u. Pharm. 113 : 47. 1860.

THE EFFECT OF DIHYDROSTREPTOMYCIN ON THE FORMATION OF ADAPTIVE ENZYMES BY A STRAIN OF *ESCHERICHIA COLI*[1]

By Susan M. Roote and W. J. Polglase

Abstract

The adaptive formation of enzymes for the oxidation of L-arabinose, lactose, and D-glucuronic acid was inhibited by dihydrostreptomycin in a strain of *Escherichia coli* which would not grow in the presence of this antibiotic. Adaptive enzyme formation was not inhibited in *E. coli* variants which would grow in the presence of dihydrostreptomycin or which required the antibiotic for growth.

Dihydrostreptomycin and Adaptive Enzymes

The inhibition by streptomycin of adaptive enzyme formation (1) was first demonstrated by Fitzgerald, Bernheim, and Fitzgerald (2). These workers observed that certain strains of mycobacteria could oxidize benzoic acid after formation of an adaptive enzyme. The formation of the adaptive enzyme and, consequently, the oxidation of benzoic acid were inhibited by streptomycin (2). It had been observed earlier by Cohen (1) that streptomycin will combine with nucleic acids. Fitzgerald *et al.* (2) suggested that this combination with nucleic acids is probably part of the mechanism by which streptomycin inhibits adaptive enzyme formation. It was concluded (2) that the inhibition of adaptive enzyme formation might be one aspect of the mechanism of the inhibition of growth by streptomycin. Umbreit has suggested (7) that the inhibition of adaptive enzyme formation could be the result of inhibition by streptomycin of some other (possibly distantly removed) metabolic process. Umbreit and co-workers have published a number of articles (4, 5, 8–11) which indicate that the site of action of streptomycin in *E. coli* may be a condensation reaction between pyruvate and oxalacetate to yield a seven-carbon phosphorylated compound (9) of significance in the terminal respiration system.

It is the purpose of this article to report the results of a study of the effect of dihydrostreptomycin on the formation of adaptive enzymes by a strain of *E. coli*.

Materials and Methods

Cultures

Three *E. coli* variants were used in this work: a streptomycin-sensitive culture, the growth of which was arrested by approximately 30 units per ml. of streptomycin; a streptomycin-resistant type which would grow in the presence of 1000 units per ml. of streptomycin; and a streptomycin-dependent (3) variant which required for growth at least 30 units per ml. of streptomycin. It was observed that any one of these variants would yield either of the other

[1] *Manuscript received April 29, 1955.*
Contribution from the Department of Biochemistry, Faculty of Medicine, University of British Columbia, Vancouver, B.C.

two under appropriate experimental conditions. The effect of dihydrostreptomycin on growth of these cultures was indistinguishable from the effect of streptomycin.

Media

A synthetic medium was used consisting of K_2HPO_4 (0.7%), KH_2PO_4 (0.3%), sodium citrate (0.05%), $MgSO_4$ (0.01%), $(NH_4)_2SO_4$ (0.1%), and a carbon source (0.2%). Resistant and dependent cultures were grown in the presence of 1000 units per ml. of dihydrostreptomycin. For respiration experiments, the cultures were grown for 20 hr. in Roux flasks. The cells were then harvested and washed with saline, by centrifuging at 2° C., and finally were suspended in saline. This suspension was adjusted to contain 0.5 mgm. of bacterial nitrogen per ml. The antibiotic was used in the form of dihydrostreptomycin sulphate in aqueous solution. Accumulated data on the dihydro form of streptomycin indicate that in its biological activity it is quantitatively equivalent to streptomycin.

Respiration experiments were performed by conventional manometric techniques using a Warburg-Barcroft apparatus.

Growth experiments were performed by inoculation into tubes of the above synthetic medium, followed by incubation at 37° C. for 24 hr., and estimation of turbidity.

Each cup contained phosphate buffer: 1.5 ml. of *M*/15, pH 7.0; dihydrostreptomycin sulphate, when present: 0.3 ml. of a solution containing 10,000 units per ml.; substrate solution: 0.2 ml. (5 micromoles); bacterial cell suspension: 0.5 ml.; and distilled water to bring the total volume to 3.00 ml. The center well contained 0.15 ml. of 20% potassium hydroxide solution. The bacterial suspensions were preincubated with streptomycin for one hour before the addition of substrate from the side arm.

Results and Discussion

Fig. 1 shows the effect of dihydrostreptomycin on the oxidation of arabinose by dihydrostreptomycin-sensitive *E. coli* grown with glucose as the carbon source. In the absence of dihydrostreptomycin, oxidation of arabinose began after a lag of about 30 min. When the antibiotic was present, this oxidation was markedly inhibited. When the streptomycin-sensitive strain was grown with arabinose as the carbon source, the harvested, washed bacterial cells oxidized arabinose without a preliminary lag period and this oxidation was only slightly affected by streptomycin (Fig. 2). Similar results were obtained with two other compounds, lactose and glucuronic acid (Table I). Thus, in dihydrostreptomycin-sensitive *E. coli*, the process of adaptation to oxidation of arabinose, glucuronic acid, and lactose was inhibited by dihydrostreptomycin.

The dihydrostreptomycin-resistant *E. coli* variant adapted rather slowly to arabinose and the process of adaptation was not significantly affected by dihydrostreptomycin (Fig. 3). The dependent variant adapted to arabinose readily and the process of adaptation was somewhat enhanced in the presence

TABLE I

DIHYDROSTREPTOMYCIN-SENSITIVE *E. coli*: OXYGEN UPTAKE IN MICROLITERS

	Substrate: lactose				Substrate: glucuronic acid			
	Grown on glucose		Grown on lactose		Grown on glucose		Grown on glucuronic	
Time, min.	−	+	−	+	−	+	−	+
10	0	3	71	62	0	0	17	14
20	0	6	141	132	0	0	64	63
30	0	11	211	209	6	1	83	81
40	8	13	284	289	19	9	99	96
50	17	15	322	336	37	16	105	108
60	30	19	357	376	54	21	120	121
70	45	27	378	411	76	28	129	125
80	66	27	415	441	97	34	134	139
90	82	34	—	—	111	41	143	149
100	110	38	—	—	126	47	145	152
110	138	42	—	—	141	52	—	—
120	161	47	—	—	155	59	—	—
130	195	54	—	—	176	60	—	—
140	205	57	—	—	190	66	—	—
150	239	68	—	—	204	—	—	—

+ = *1000 units/ml. of dihydrostreptomycin.*
− = *No dihydrostreptomycin.*

of streptomycin (Fig. 4). It is not possible to determine the behavior of dependent cells in the complete absence of dihydrostreptomycin, since during growth the cells probably absorb a certain amount of the drug which cannot be removed by washing.

Table II shows that dihydrostreptomycin-resistant and dependent *E. coli* cells form adaptive enzymes to two other compounds, lactose and D-glucuronic acid, either in the presence or in the absence of dihydrostreptomycin.

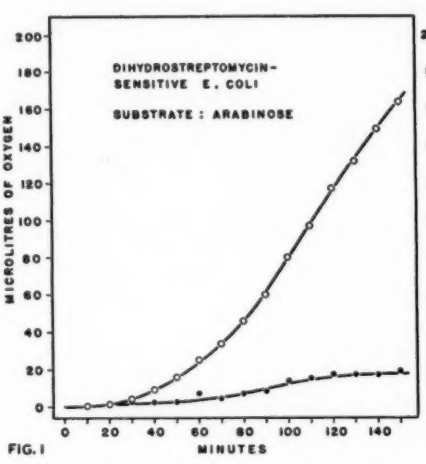
TABLE II

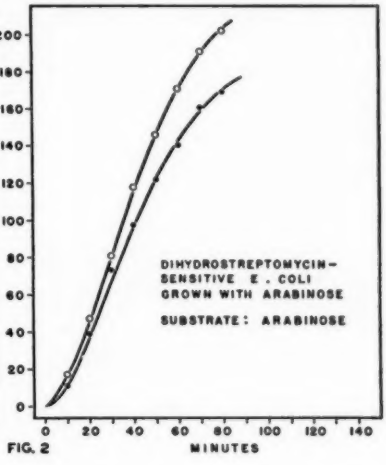
OXYGEN UPTAKE IN MICROLITERS

	Dihydrostreptomycin-resistant *E. coli*				Dihydrostreptomycin-dependent *E. coli*			
	Substrate: lactose		Substrate: glucuronic acid		Substrate: lactose		Substrate: glucuronic acid	
Time, min.	−	+	−	+	−	+	−	+
10	0	2	0	0	4	5	0	0
20	0	1	0	6	4	8	2	12
30	0	2	6	1	20	20	20	20
40	0	5	14	12	55	66	48	44
50	0	6	27	24	95	109	57	56
60	6	8	39	38	145	145	90	81
70	11	11	53	53	192	211	103	103
80	20	18	68	68	248	277	117	114
90	26	22	76	79	307	352	130	125
100	35	28	89	89	368	392	138	134
110	43	39	99	103	430	473	143	140
120	60	49	111	112	475	527	153	150
130	74	61	121	124	—	—	161	153
140	91	77	131	135	—	—	—	—
150	105	90	148	145	—	—	—	—

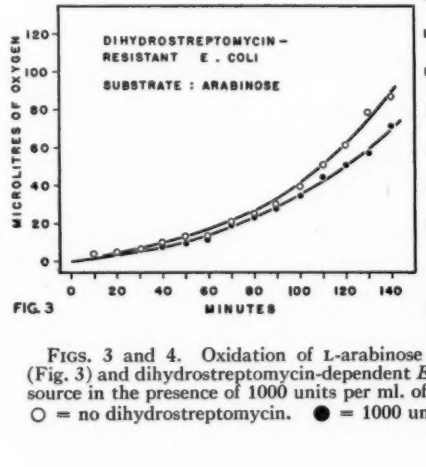
+ = *1000 units/ml. of dihydrostreptomycin.*
− = *No dihydrostreptomycin.*
Both cultures were grown with glucose as carbon source.

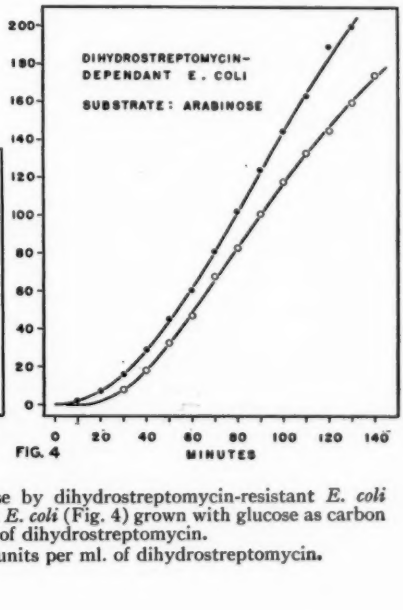
The foregoing results establish that the ability of a microorganism to develop adaptive enzymes in the presence of streptomycin is related to the growth-sensitivity of the organism to streptomycin.





FIGS. 1 and 2. Oxidation of L-arabinose by dihydrostreptomycin-sensitive *E. coli* grown with glucose (Fig. 1) and L-arabinose (Fig. 2) as carbon source.
○ = no dihydrostreptomycin. ● = 1000 units per ml. of dihydrostreptomycin.





FIGS. 3 and 4. Oxidation of L-arabinose by dihydrostreptomycin-resistant *E. coli* (Fig. 3) and dihydrostreptomycin-dependent *E. coli* (Fig. 4) grown with glucose as carbon source in the presence of 1000 units per ml. of dihydrostreptomycin.
○ = no dihydrostreptomycin. ● = 1000 units per ml. of dihydrostreptomycin.

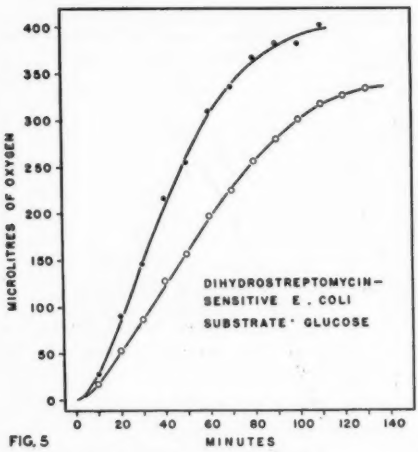


FIG. 5

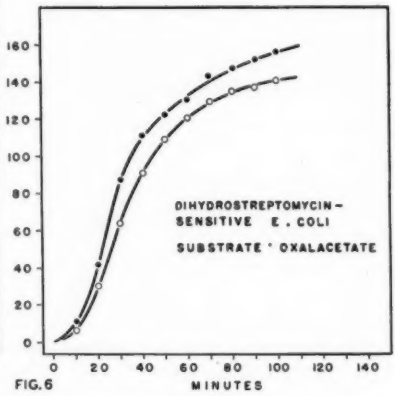


FIG. 6

FIGS. 5 and 6. Oxidation of glucose (Fig. 5) and oxalacetate (Fig. 6) by dihydrostreptomycin-sensitive *E. coli* grown with glucose as carbon source.
○ = no dihydrostreptomycin. ● = 1000 units per ml. of dihydrostreptomycin.

The dihydrostreptomycin-sensitive *E. coli* which had been grown on glucose readily oxidized glucose and also oxalacetate. Oxidation of these substrates was enhanced rather than inhibited by dihydrostreptomycin (Figs. 5 and 6).

The data obtained are interpreted to indicate that, of the metabolic processes examined, only the process of adaptive enzyme formation has been found to be related consistently to effects of dihydrostreptomycin on growth. Since oxygen uptake is a relatively non-specific, complex reaction, measurement of the formation of individual enzyme systems is in progress.

Acknowledgments

The *Escherichia coli* culture used in this work was originally obtained as a streptomycin-dependent strain from Dr. Thomas F. Paine, Jr., Massachusetts General Hospital. Streptomycin and dihydrostreptomycin were kindly supplied by Merck & Co., Ltd., Montreal. This work was supported by a grant from the Defence Research Board of Canada (DRB 368).

References

1. COHEN, S. S. J. Biol. Chem. 166 : 393. 1946.
2. FITZGERALD, R. J., BERNHEIM, F., and FITZGERALD, D. B. J. Biol. Chem. 175 : 195. 1948.
3. MILLER, C. P. and BOHNHOFF, M. Science, 105 : 620. 1947.
4. OGINSKY, E. L., SMITH, P. H., and UMBREIT, W. W. J. Bacteriol. 58 : 761. 1949.
5. SMITH, P. H., OGINSKY, E. L., and UMBREIT, W. W. J. Bacteriol. 58 : 761. 1949.
6. STANIER, R. Y. J. Bacteriol. 54 : 339. 1947.
7. UMBREIT, W. W. J. Biol. Chem. 177 : 703. 1949.
8. UMBREIT, W. W. Trans. N. Y. Acad. Sci. 15 : 8. 1952.
9. UMBREIT, W. W. J. Bacteriol. 66 : 74. 1953.
10. UMBREIT, W. W., SMITH, P. H., and OGINSKY, E. L. J. Bacteriol. 61 : 595. 1951.
11. UMBREIT, W. W. and TONHAZY, N. E. J. Bacteriol. 58 : 769. 1949.

A NOTE ON THE EFFECT OF AUREOMYCIN ON THE RESPONSE OF THE RAT TO VITAMIN D[1]

By T. K. Murray and J. A. Campbell

Abstract

The response of rachitic rats to doses of vitamin D, as measured by the line test, was increased by the addition of aureomycin to the rachitogenic diet. The weight gain of these animals was also increased by the antibiotic. In the absence of vitamin D aureomycin had no effect on either calcification or weight gain.

It has been reported (5) that penicillin increased calcification in chicks when low levels of vitamin D were fed, while at higher levels of vitamin D aureomycin was without effect. Migicovsky *et al.* (2) found that 30 p.p.m. of penicillin in the feed increased the Ca^{45} content of the tibia of chicks by an average of 70%. Rekling, Gjeddeback, and Storling (4) reported that the oral administration of penicillin increased the effectiveness of vitamin D_3 in promoting calcification in young rats but did not itself prevent rickets. In this laboratory aureomycin has been found (3) to increase the apparent utilization of vitamin A, and in the course of routine assays the opportunity was afforded to test the effect of this antibiotic on the response of the rachitic rat to graded doses of vitamin D.

The U.S.P. XIV (6) rat line test for vitamin D was modified by the inclusion of 5% brewers' yeast in the rachitogenic diet and by the use of three levels of vitamin D for both the reference and sample. The total doses amounted to 0.75, 1.5, or 3 I.U. and were administered orally, by syringe, over a period of six days. Rats were killed on the third day after the final dose. Eight to 10 rats of each sex were used in each group.

In a series of 10 assays, three groups which received doses of the reference standard were compared with three similar groups which received the same doses but which also received aureomycin in their diet at the rate of 66 mgm./kgm. The aureomycin was added to the diet two days before dosing and was continued until the end of the test, a total of 11 days. At this time the extent of calcification in these groups was compared with that of the control groups, by usual bio-assay procedures (1). Weight gains during the assay period were also recorded and compared.

A summary of the results is shown in Table I. It may be noted that although aureomycin increased calcification in each assay, the increase was within the confidence limits of the assay in every case. When the assays were combined by the method of Bliss (1), however, the average increase in calcification was 18.0% with confidence limits of $\pm$ 11.2%. The response

[1] *Manuscript received April 28, 1955.*

Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada.

TABLE I

THE EFFECT OF AUREOMYCIN ON THE CALCIFICATION AND WEIGHT GAIN OF RATS IN THE VITAMIN D LINE TEST

Assay	Increase in calcification, %	Confidence limits ($P = 0.05$), %	Mean wt. gains, gm.	
			No aureomycin	Aureomycin
1	16.5	± 51.0	2.8	4.1
2	38.1	44.7	4.2	6.5
3	4.2	28.0	5.6	7.2
4	6.2	46.7	4.8	6.6
5	19.9	45.4	3.7	7.2
6	18.7	35.2	4.0	6.9
7	3.7	38.9	4.8	5.4
8	19.1	37.3	7.1	7.2
9	32.3	41.6	2.5	2.9
10	21.1	39.6	4.9	5.9
Means	18.0	11.2	4.4	6.0

to aureomycin was therefore significant at $P = 0.05$. The response lines were essentially parallel but showed some tendency to converge at the highest level of vitamin D.

Aureomycin increased the mean weight gains during the nine-day periods of each assay. An analysis of variance of all growth data (984 rats) is shown in Table II. It will be noted that highly significant differences exist between assays, between sexes, and between the aureomycin and the control groups. None of the individual interactions was significantly greater than the residual error at the 5% probability level. When pooled, they just reached significance.

It is interesting to note that weight gains were not influenced by the level of vitamin D. It would therefore appear that aureomycin did not influence growth rate by increasing the absorption of vitamin D. Unpublished results in this laboratory indicate that in the absence of vitamin D, aureomycin had no effect on either growth or calcification during the three week period between weaning and assay. This observation would seem to eliminate increased food consumption as a factor and is in agreement with the results reported by Rekling *et al.* (4). Ross and Yacowitz (5) found that chicks which did not receive vitamin D did not respond to penicillin with increased growth unless extra phosphorus was added to the diet. This possibility has not been investigated here.

TABLE II

ANALYSIS OF VARIANCE OF GROWTH OF RATS ON VITAMIN D ASSAY IN THE PRESENCE AND ABSENCE OF AUREOMYCIN

Source of variation	Degrees of freedom	Mean square	*F*
Between 10 assays	9	135.7	7.6†
Between two sexes	1	952.0	53.2†
Between three levels	2	6.7	0.5
Between two treatments	1	414.0	23.1†
Interactions	106	17.9	1.4*
Residual	894	12.7	—
	983		

* *Significant at P = 0.05.*
† *Significant at P = 0.01.*

The results reported here indicate that aureomycin increased both the degree of calcification and the weight gain of rats receiving a rachitogenic diet and graded doses of vitamin D. The increased weight gain did not appear to be a result of increased absorption of vitamin D.

Acknowledgments

The authors wish to acknowledge the assistance of Mr. N. T. Gridgeman in the statistical analysis of the data and of Mrs. Marion Shama in carrying out the assays.

References

1. BLISS, C. I. and GYORGY, P. Vitamin methods. Vol. II. Academic Press, Inc., New York. 1951.
2. MIGICOVSKY, B. B., NIELSON, A. M., GLUCK, M., and BURGESS, R. Arch. Biochem. and Biophys. 34 : 479. 1951.
3. MURRAY, T. K. and CAMPBELL, J. A. J. Nutrition. In press. 1955.
4. REKLING, E., GJEDDEBACK, N. F., and STORLING, E. Nord. Med. 51 : 300. 1954.
5. ROSS, E. and YACOWITZ, H. Poultry Sci. 31 : 933. 1952.
6. UNITED STATES PHARMACOPEIA. XIV. Mack Printing Company, Easton, Pa. 1950. p. 787.

RENAL RESPONSES TO HYPERVOLEMIA IN THE DOG[1]

By I. E. Young[2], J. W. Pearce,[3] and J. A. F. Stevenson

Abstract

Kidney function studies and cardiovascular measurements were made on anesthetized dogs rendered hypervolemic with infusions of dextran, plasma, whole blood, or saline. The infusions, equal in volume to the calculated initial blood volume, were given at a standard rate except those of dextran which were infused at a faster and at a slower rate. A diuresis resulted from all infusions except that of whole blood; a greater diuresis accompanied slow infusions of dextran compared with the effect of more rapid infusions of dextran of similar volume. With all infusions, except that of saline, there was no increase in glomerular filtration rate. It is inferred that part of the diuresis with dextran infusions, as well as that with plasma infusions, was due to alteration in tubular function. The RBF was normal or decreased during all infusions; it is reasoned that renal afferent vasoconstrictor activity increased during hypervolemia produced by infusions other than saline. Some possible factors in the production of the diuresis, other than the volume increase alone, are discussed.

Introduction

It has been reported (9) from this laboratory that in dogs large infusions of dextran produce a diuresis, but no increase in glomerular filtration rate or renal blood flow. A decrease in oxygen content of the blood (1, 21), increase of the plasma volume (23), and the osmotic property of the renal-excretable dextran (7, 22) have each been reported to produce a diuresis without increasing the filtration rate or renal blood flow. The role of the kidney in the maintenance of constant blood and extracellular fluid volumes, particularly when these volumes are varied without change in the tonicity, is not clear. Further investigations of kidney function during acute expansion of the blood volume with isotonic solutions are necessary. The present investigation extends the series using dextran, and includes a study of the effects of infusions of plasma, saline, and whole blood.

Methods

Renal and cardiovascular measurements were made on 26 dogs before and during the infusion of a blood volume expander. The dogs were anesthetized with sodium pentobarbital (35 mgm./kgm.). A laparotomy was done immediately and a saline-filled catheter inserted into each ureter. The incision was closed and the urine collected directly from each ureter into graduated centrifuge tubes. A catheter was inserted into the brachial artery for the collection of arterial blood samples. A second catheter was passed down the right external jugular vein into the right atrium and mean right auricular pressure (RAP) was recorded from a saline manometer connected to this catheter.

[1] *Manuscript received April 13, 1955.*
Contribution from the Department of Physiology, University of Western Ontario, London, Ontario. This work was supported by Grant No. 298 of the Defence Research Board of Canada.
[2] *Present address: Department of Bacteriology and Immunology, University of Western Ontario, London, Ontario.*
[3] *National Research Council Senior Fellow.*

For the measurement of renal clearances the blood concentrations of creatinine (15 mgm. %) and *p*-aminohippurate (PAH) (2 mgm. %) were established by a priming injection of these substances in normal saline. Constant blood levels were maintained throughout the experiment by a slow intravenous drip.

The amount for the priming injections was calculated as follows:

$$\text{Priming injection (mgm.)} = \begin{bmatrix}\text{weight of animal (kgm.)}\end{bmatrix} \begin{bmatrix}\dfrac{\text{volume of distribution (\% weight)}}{100}\end{bmatrix} \begin{bmatrix}\text{intended plasma concentration (mgm./ml.)}\end{bmatrix} [1000]$$

A volume of distribution of 50% was used for creatinine and 30% for PAH (10). The concentration of the sustaining intravenous infusion was calculated as:

$$\text{Conc. in sustaining infusion (mgm./ml.)} = \frac{\begin{bmatrix}\text{Plasma conc. (mgm./ml.)}\end{bmatrix} \begin{bmatrix}\text{Estimated clearance (ml./min.)}\end{bmatrix}}{[\text{Rate of infusion (ml./min.)}]}$$

The estimated clearances for creatinine (4.29 ml./kgm./min.) and PAH (13.5 ml./kgm./min.) were based on values quoted by Smith (19). The rate of the infusion was 1.5 ml./min. for the control periods and 1.8 ml./min. during the infusion of the blood volume expander.

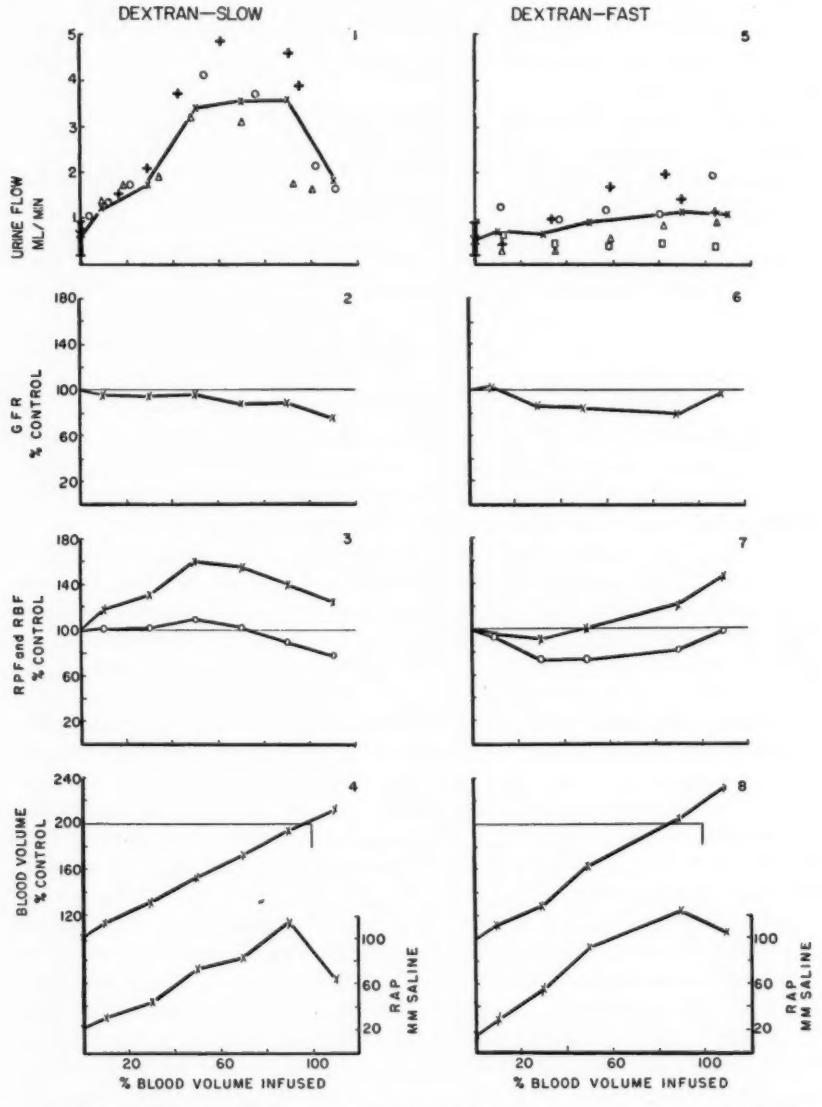
Two control values for the 15 min. clearances of creatinine and PAH, the hematocrit value, and the RAP were determined at two and one-half and three hours after the beginning of anesthesia. A blood volume expander (dextran*, plasma, saline, or whole blood), warmed to 37° C., was then infused into the femoral vein. The rate was usually 1.0 ml./kgm./min., but in two dextran series a slower (0.5 ml./kgm./min.) and a faster (2.0 ml./kgm./min.) rate were used. The infusion continued until an amount approximately equivalent to the calculated blood volume (85 ml./kgm.) had been infused. Renal clearances were determined by the collection of urine over consecutive 10 or 15 min. periods during the infusion. Blood samples were withdrawn at the mid-point of each clearance period and the clearances calculated by the standard UV/P formula. The RAP was recorded at the mid-point of each clearance period.

Compatible whole blood for infusion was collected immediately prior to the experiment from heparinized anesthetized donor dogs. Plasma was obtained from whole blood collected similarly and centrifuged.

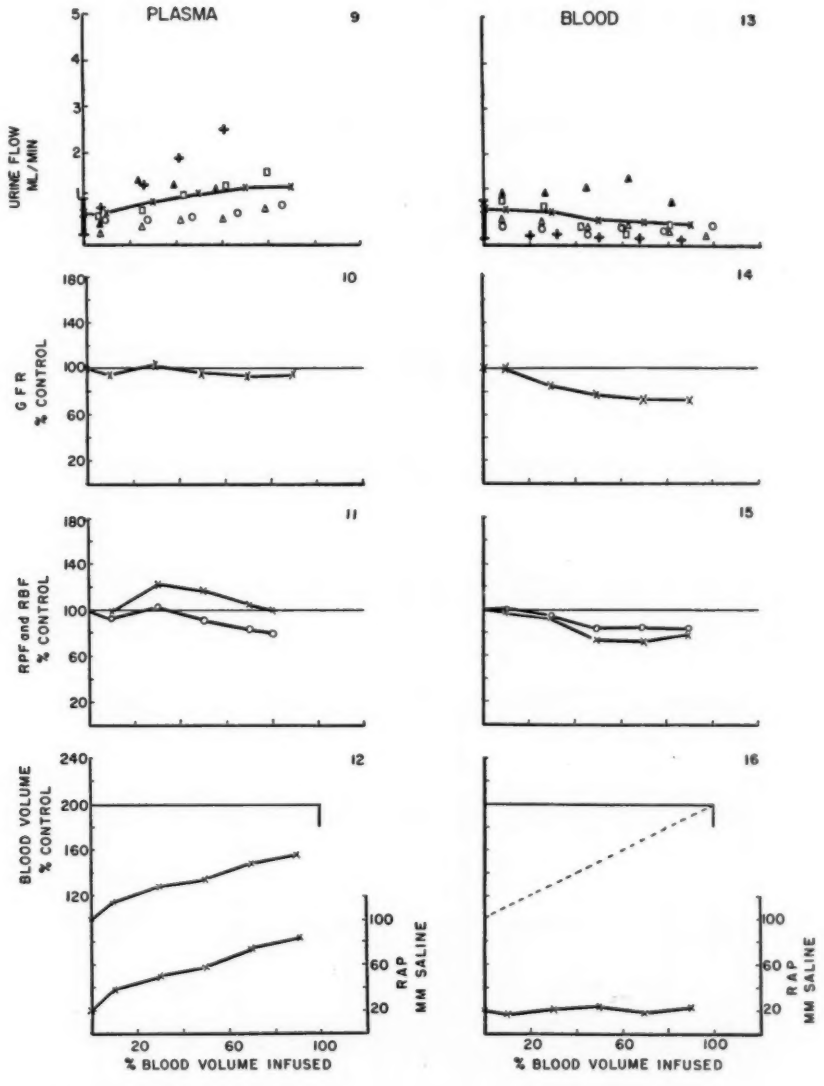
PAH was determined by a modification of the method of Smith, Finkelstein, Aliminosa, Crawford, and Graber (20). A modification of the method of Bonsnes and Taussky (2) was used for the determination of creatinine. The hematocrit values were obtained from heparinized arterial blood samples spun in Wintrobe tubes for 30 min. at 3000 r.p.m.

The renal clearance of creatinine was considered equivalent to the glomerular filtration rate (GFR) and the clearance of PAH to the effective renal

* *'Intradex'—6% solution of dextran in saline, kindly supplied by Glaxo (Canada) Ltd.*



FIGS. 1-16. Mean values of urine flow, glomerular filtration rate (GFR), renal plasma flow (RPF —×—), renal blood flow (RBF —×—), calculated blood volume, and right auricular pressure (RAP) plotted against the infused volume expressed as a percentage of estimated initial blood volume. The urine flow and RAP are expressed in absolute units,



FIGS. 1-16—*Concluded*

and the other indices as percentages of their mean control values. The symbols in Figs. 1, 5, 9, 13 represent urine flow values for individual dogs. The line bar at the ordinate represents the spread of the control values for each series.

TABLE I *A*

Time, min.	GFR, ml./min.	RPF, ml./min.	RBF, ml./min.	U.F., ml./min.	Hct, %	RAP, mm. saline	Sp. gr.	Inf. vol., % B.V.	B.V., %	% Inc. MCHC
Dextran infusion at 1.0 ml./kgm./min. (14.3 kgm., female)										
0– 15	55.0	152	339	0.69	57.5	35				
30– 45	64.7	170	401	1.39	57.5	40				
Infusion begins										
45– 60	52.3	138	260	1.26	47.0	45	—	27	122	—
60– 75	69.8	253	411	3.93	38.5	70	—	47	149	—
75– 90	63.6	274	412	8.18	33.5	90	—	66	171	—
90–105	55.1	232	332	7.49	30.0	100	—	84	192	—
105–120	56.5	228	310	7.00	26.5	73	—	100	224	—
Dextran infusion at 0.5 ml./kgm./min. (10.2 kgm., female)										
0– 15	45.8	112	239	0.40	53.5	25	1025			
30– 45	48.1	131	285	0.67	54.0	22	1025			
Infusion begins										
45– 60	46.7	152	266	1.02	43.0	33	1025	13	118	5.2
60– 75	45.2	165	266	1.30	38.0	42	1026	21	131	7.2
75– 90	48.7	156	236	1.68	34.0	42	1027	30	146	7.8
90–105	48.7	214	295	4.16	27.5	73	1027	53	170	13.0
105–120	41.5	203	268	3.68	24.0	60	1027	76	197	12.3
120–135	33.0	145	182	2.16	21.0	47	1028	102	215	16.2
135–150	32.9	155	197	1.64	21.0	47	1029	110	215	15.9
Dextran infusion at 2.0 ml./kgm./min. (9.5 kgm., female)										
0– 15	39.4	103	212	0.19	49.0	15	1025			
30– 45	48.6	114	228	0.22	50.0	12	1024			
Infusion begins										
45– 55	50.8	100	176	0.30	43.0	22	1025	12	114	2.7
55– 65	41.1	109	166	0.33	34.0	60	1025	35	141	4.5
65– 75	42.5	80	109	0.50	26.5	105	1026	59	176	6.7
75– 85	36.2	148	193	0.77	23.0	135	1027	82	206	6.0
85– 95	43.4	156	195	0.89	19.5	125	1028	105	230	10.3

TABLE I *B*

Time, min.	GFR, ml./min.	RPF, ml./min.	RBF, ml./min.	U.F., ml./min.	Hct, %	RAP, mm. saline	Sp. gr.	Inf. vol., % B.V.	B.V., %	% Inc. MCHC
Plasma infusion at 1.0 ml./kgm./min. (9.3 kgm., male)										
0– 15	50.0	114	235	0.26	51.8	38	1025			
30– 45	54.6	98	207	0.16	52.9	38	1026			
Infusion begins										
45– 60	45.5	106	188	0.23	43.1	62	1025	8	122	1.1
60– 75	54.4	166	271	0.39	38.9	80	1024	26	136	0.7
75– 90	44.3	146	237	0.50	38.2	42	1025	43	138	1.3
90–105	50.7	148	234	0.52	36.6	58	1025	61	145	1.3
105–120	52.6	121	182	0.76	33.6	90	1025	79	157	1.3
Whole blood infusion at 1.0 ml./kgm./min. (9.1 kgm., male)										
0– 15	30.7	72.0	131	0.18	45.0	12	1025			
30– 45	32.9	92.4	172	0.33	47.5	12	1025			
Infusion begins										
45– 60	33.6	91.5	174	0.51	47.5	10	1025	8	—	−1.8
60– 75	33.2	85.5	158	0.48	46.0	10	1026	26	—	−0.6
75– 90	29.0	66.4	122	0.37	46.0	10	1025	44	—	−2.1
90–105	30.1	57.9	106	0.35	45.5	12	1025	62	—	−0.6
105–120	30.4	51.8	98	0.28	47.0	10	1025	80	—	−0.6
Normal saline (0.85%) infusion at 1.0 ml./kgm./min. (14.8 kgm., female)										
0– 15	48.7	108	193	0.33	44.0	8				
30– 45	42.9	130	236	0.93	45.2	4				
Infusion begins										
45– 60	72.4	192	340	1.10	43.5	5	—	8	129	−0.3
60– 75	50.0	105	178	1.03	41.2	6	—	24	136	−1.8
75– 90	45.2	138	227	1.33	40.2	7	—	41	139	−4.6
90–105	48.8	130	213	1.51	39.0	7	—	58	144	−3.0
105–120	42.2	101	160	1.59	36.7	20	—	75	153	1.5
120–135	43.5	99	150	1.87	34.1	42	—	91	164	1.2

plasma flow (RPF). The effective renal blood flow (RBF) was calculated from the RPF and arterial hematocrit values. In the dextran, plasma, and saline infusion experiments an estimate of the whole blood volume increase was derived from the fall in hematocrit, corrected for any change in mean corpuscular hemoglobin concentration (MCHC). Such an estimate is open to the criticism that one assumes that no change occurred in total number of circulating red cells. However, it was considered a more valid approximation of the true blood volume increase than the estimate derived from the amount of expander actually infused.

Results

With the slow (0.5 ml./kgm./min.—three dogs) infusion of dextran the average urine flow increased about sevenfold (Fig. 1) but barely doubled during the fast (2.0 ml./kgm./min.—four dogs) infusion (Fig. 5). The points represent the values from individual experiments which contribute to the mean line.

Figs. 2 and 3 show the effect of a slow, and Figs. 6 and 7 of a rapid infusion of dextran on the GFR, RPF, and RBF. For each dog the value of a function was expressed as a percentage of the mean control value of that function and the mean of these percentages is plotted against the volume infused expressed as a percentage of the calculated blood volume. It can be seen that there was no marked change in GFR with either rate of infusion, but the line joining the mean values for each group was at all times below control level. There was a considerable increase in RPF during the slow infusion, but little increase in this function except towards the end of the fast infusion. The mean RBF was normal or low-normal during both rates of infusion.

The increase in whole blood volume in both groups was greater than expected from the volume infused (Figs. 4, 8). The right auricular pressure rose sharply reaching peak mean values of 116 mm. of saline during the slow (Fig. 4) and 108 mm. during the fast infusion (Fig. 8). It fell gradually after the infusion ended.

The results shown here for the slow infusion of dextran were in all ways similar to the results previously reported (15 dogs) for the infusion of dextran at 1.0 ml./kgm./min. (9) and which have been confirmed in five additional dogs of this series infused at this same rate (Table 1 *A*). The only apparent difference, therefore, between the effects of the fast and slow infusions, is the striking discrepancy in the volume of urine excreted.

It is to be noted that, during the infusion of plasma (1 ml./kgm./min.—five dogs), there was no change in GFR (Fig. 10). There was, however, a progressive slight decrease in RBF (Fig. 11). On the other hand both the GFR and RBF decreased during the infusion of whole blood (1 ml./kgm./min.—five dogs) (Figs. 14, 15).

An increase in urine flow from 0.63 ml./min. to 1.28 ml./min. occurred during the infusion of plasma (Fig. 9). There was no increase in urine flow during the infusion of whole blood (Fig. 13). In another study by one of

us (J.W.P.) four of five dogs under morphine–chloralose anesthesia also failed to show a diuresis during the infusion of whole blood.

The mean RAP rose during the infusion of plasma from 26 mm. to 74 mm. of saline (Fig. 12), but remained remarkably constant during the infusion of whole blood (Fig. 16). Indeed, in the latter instance, the mean pressure did not vary from a control value of 21 mm. of saline by more than 4 mm.

Expansion of the extracellular fluid volume with normal saline (1.0 ml./kgm./min.—four dogs) produced the expected increase in GFR and urine flow. The increase in blood volume was, of course, less than the amount of saline infused (Table I *B*). The mean RAP rose slightly, from 12 mm. to 38 mm. of saline.

Detailed protocols typical of each series are presented in Tables I *A* and I *B*.

Post-mortem examination, carried out on all the dogs, revealed no pulmonary edema but varying degrees of ascites and engorgement of abdominal viscera.

Discussion

Previous studies indicate that expansion of the plasma volume produces a diuresis (14, 23). It was considered in an earlier publication (9) that the marked diuresis observed during the infusion of dextran could be explained as an osmotic or proximal diuresis due to the renal-excretable dextran, but the experiments reported here suggest that some other mechanism is also effective in the production of the increased urine flow. The infusion of the same volume of dextran at three different rates (0.5, 1.0, and 2.0 ml./kgm./min.) produced similar changes in RAP, renal hemodynamics, and total blood volume expansion. The increase in urine flow, however, was much less at the fastest than at the two slower rates. There is no evidence that the GFR changed significantly in any of the series. Brewer (3) established that dextran is excreted solely by glomerular filtration and, therefore, the number of osmotic particles of dextran in the tubular urine would be comparable in these experiments for any given increase in blood volume regardless of the rate of infusion. Petersdorf and Welt (17) have suggested that a very rapid increase in the oncotic pressure of the plasma can promote secretion of the antidiuretic hormone (ADH). The dextran used was hyperosmotic to dog plasma (13); its rapid infusion presumably caused a sharp increase in the osmotic pressure of the blood and thus may have stimulated the release of ADH. This hormone does not reduce an osmotic diuresis originating in the proximal tubule. Although the same degree of proximal osmotic diuresis presumably existed at all rates of dextran infusion, the smaller increase in urine flow during the fast infusion suggests that during the slow infusions there was distal diuresis in addition to the proximal osmotic diuresis. This would agree with the concept that expansion of the plasma volume in some way inhibits the release of ADH (23, 8).

A diuresis could also result from an increased excretion of salt due to inhibition of mineralocorticoid secretion. It has been suggested that expansion of the plasma volume can increase the urinary excretion of sodium (18). A

decrease in both adrenocortical and neurohypophyseal effects would be beneficial in such circumstances and there is as yet no apparent reason why this double inhibition could not occur.

Whole plasma was infused in an attempt to confirm the presence of a distal diuresis with expansion of the plasma volume. No colloidal osmotic particles could be considered to contribute to the diuresis observed unless there was an inhibition of reabsorption or an excretion of electrolytes in the tubule. There was, as with the dextran infusions, no alteration in the GFR, but an increase in urine flow did occur.

An expansion of blood volume would appear to be insufficient to evoke a diuresis, as the infusion of whole blood produced no change in urine flow under the conditions of these experiments. There does not appear to have been an immediate relocation of fluid as there was no change in plasma specific gravity, hematocrit, or MCHC (Tables I *A* and I *B*). Adrenaline, which in large amounts could produce an antidiuresis, was not present in such quantities in the infused blood for there were no marked rises in arterial blood pressure. ADH could have been present in the infused blood. However, the amount infused would have been greater in the plasma experiments and a diuresis was observed.

With the infusion of whole blood there was an absence, not only of diuresis, but also of any increase in RAP, whereas with the other infusates diuresis was always associated with an increase in RAP. When the RAP increases with a rapid infusion, the left auricular pressure increases also, but to a greater extent (15). It is probably safe to assume an increased right, and therefore, left atrial volume corresponding to such a pressure rise. The failure of the RAP to rise during the massive whole blood infusion would thus imply that there was no increase in atrial volumes. Gauer, Henry, Sieker, and Wendt (8) and Henry *et al.* (12) have shown that maneuvers designed to increase the thoracic blood volume or the left atrial volume alone usually produce an increased urine flow. It has also been shown that such increases in left atrial volume can be registered by atrial receptors with fibers in the vagus nerve (16). Henry *et al.* (11) have suggested that increased activity in these fibers may initiate the diuresis and should this suggestion prove correct, the difference in diuretic response to blood and other infusates could be explained.

Other factors which, alone or in conjunction with simple hypervolemia, could produce an increased urine flow should be considered. Dextran and plasma are both non-oxygen carrying substances, yet it is unlikely that the considerable decrease in blood oxygen content (especially during dextran infusion) was responsible for the diuresis, for the cardiac output is markedly increased with the infusion of dextran and the total arterial oxygen transport remains relatively constant in spite of the greatly decreased hematocrit (9). That the diuresis was due to anoxia of the kidney seems unlikely for the kidney is relatively insensitive to oxygen lack (6). This does not, however, eliminate the possibility that the decrease in oxygen supply to some other area might be effective in the reflex production of a diuresis (4, 5).

There was a slight reduction in GFR, RPF, RBF with the infusion of whole blood. Since there was no marked change in the hematocrit there was probably no change in viscosity, and therefore, a renal afferent vasoconstriction probably had occurred.

One might expect an increased GFR to be one of the renal adjustments to a sharply increased blood or plasma volume. The experiments presented here indicate that only with the infusion of saline (when the total extracellular fluid volume is increased) was there an increase in GFR, and this may be a species peculiarity (19).

The failure of the RBF to increase during hypervolemia, and indeed the trend for it to fall below normal, imply that the renal vessels could not have taken part in the general vasodilatation necessary to maintain a relatively constant blood pressure in the face of a much increased cardiac output (9). This selective maintenance of caliber, and in some cases decrease in caliber, probably required increased vasoconstrictor activity in the kidney. The absence of any increase in GFR would, furthermore, imply that any such vasoconstriction must have involved the afferent rather than the efferent arteriole.

Summary and Conclusions

1. Renal and cardiovascular measurements were made in anesthetized dogs before and during acute expansion of the blood volume by the infusion of dextran, plasma, whole blood, or saline.

2. The GFR was normal or slightly decreased during all infusions except that of saline. The RPF was increased with all infusions except that of whole blood, which produced a decrease.

3. The calculated RBF was normal or decreased during all infusions. It is reasoned that renal afferent vasoconstrictor activity increased during hypervolemia produced by infusions other than saline.

4. The marked diuresis produced by slow infusions of dextran (0.5 ml./kgm./min.) can be explained as due to inhibition of ADH as well as to the osmotic action of the renal-excretable fraction of dextran. The diuresis resulting from plasma infusion must be explained as a result of change in tubular function, as there was no increase in GFR.

5. The lack of any diuresis during the infusion of whole blood is unexplained, but suggests that blood volume expansion alone is not sufficient stimulus for the diuretic mechanism.

6. Hypoxemia and elevated RAP, in addition to expansion of plasma volume, are discussed as possible stimuli for the diuresis. Possible neuroendocrine mechanisms of the diuresis are considered.

Acknowledgments

Thanks are extended to Rae Joan Burgess for her careful technical assistance.

References

1. BERGER, E. Y., GALDSTON, M., and HORWITZ, S. A. J. Clin. Invest. 28 : 648. 1949.
2. BONSNES, R. W. and TAUSSKY, H. H. J. Biol. Chem. 158 : 581. 1945.
3. BREWER, D. B. Proc. Roy. Soc. Med. 44 : 561. 1951.
4. BRIGGS, A. P., FOWELL, D. M., HAMILTON, W. F., REMINGTON, J. W., WHEELER, N. C., and WINSLOW, J. A. J. Clin. Invest. 27 : 810. 1948.
5. BULL, G. M. Visceral circulation. J. & A. Churchill, Ltd., London. 1952. p. 242.
6. DOLE, V. P., EMERSON, K., JR., PHILLIPS, R. A., HAMILTON, P., and VAN SLYKE, D. D. Am. J. Physiol. 145 : 337. 1946.
7. FLEMING, J. W., CARGILL, W. H., and BLOOM, W. L. Proc. Soc. Exptl. Biol. Med. 79 : 3. 1952.
8. GAUER, O. H., HENRY, J. P., SIEKER, H. O., and WENDT, W. E. J. Clin. Invest. 33 : 287. 1954.
9. GOWDEY, C. W. and YOUNG, I. E. Can. J. Biochem. Physiol. 32 : 559. 1954.
10. GREENBERG, J., SCHWARTZ, I. L., SPINNER, M., SILVER, L., and STARR, M. Am. J. Physiol. 168 : 86. 1952.
11. HENRY, J. P., GAUER, O. H., and REEVES, J. L. Am. J. Physiol. 179 : 644. 1954.
12. HENRY, J. P., GAUER, O. H., and REEVES, J. L. To be published.
13. MERRIMAN, J. E., YOUNG, I. E., and GOWDEY, C. W. Federation Proc. 13 : 387. 1954.
14. METCALFE, W. J. Clin. Invest. 23 : 403. 1944.
15. OPDYKE, D. F., DUOMARCO, J., DILLON, W. H., SCHREIBER, H., LITTLE, R. C., and SEELEY, R. D. Am. J. Physiol. 154 : 258. 1948.
16. PEARCE, J. W. and HENRY, J. P. Federation Proc. 13 : 109. 1954.
17. PETERSDORF, R. G. and WELT, L. G. J. Clin. Invest. 32 : 283. 1953.
18. SELKURT, E. E. Physiol. Revs. 34 : 287. 1954.
19. SMITH, H. W. The kidney, structure and function in health and disease. Oxford University Press, New York. 1951. pp. 333, 568.
20. SMITH, H. W., FINKELSTEIN, N., ALIMINOSA, L., CRAWFORD, B., and GRABER, M. J. Clin. Invest. 24 : 388. 1945.
21. STICKNEY, J. C., NORTHRUP, D. W., and VAN LIERE, E. J. Am. J. Physiol. 147 : 616. 1946.
22. WASSERMAN, K. and MAYERSON, H. S. Am. J. Physiol. 171 : 218. 1952.
23. WELT, G. and ORLOFF, J. J. Clin. Invest. 30 : 751. 1951.

OBSERVATIONS ON THE ACTION OF HEXAMETHONIUM BROMIDE IN TOURNIQUET SHOCK IN RATS[1]

By W. E. G. A. Spoerel

Abstract

Rats subjected to tourniquet shock were treated with hexamethonium bromide (C_6), a ganglionic blocking agent. The effect of this agent on survival, blood pressure, fluid loss, and hemoconcentration was studied. Hemoglobin as determined by the cyanhemoglobin method was used as an indicator of hemoconcentration. The fluid loss into the injured hindlegs was measured by the estimation of the fluid content of muscle tissue. The survival rate was significantly improved by administration of C_6 at the time the tourniquets were released. Treatment with C_6 two and four hours after tourniquet release provided little or no protection. No difference in fluid loss could be detected between treated and untreated rats, but the hemoconcentration was less in treated animals and followed a different pattern. Treated rats showed no blood pressure response to the release of the tourniquets. The protection provided by C_6 is thought to be due mainly to its effect on the general circulation.

Freeman and co-workers (5) were the first to demonstrate that animals with a total sympathectomy are less susceptible to hemorrhagic and traumatic shock. Similar results were obtained by treatment of animals with sympatholytic and adrenolytic drugs before an experimental shock procedure. Dibenamine and dibenzyline proved to be highly protective against irreversible shock (2, 14, 17). Tetraethylammonium chloride, a ganglionic blocking agent, also gave favorable results (6). Animals treated with these drugs before the shock procedure were able to withstand a period of severe hypotension longer than untreated rats or survived traumatic procedures which were fatal to the majority of untreated animals. Wiggers (16) emphasized that "the search for the most effective sympathetic blocking agent and the most favorable time of its administration deserves prompt attention."

If a sympathetic blockade protects against irreversible shock, hexamethonium, a potent ganglionic blocking agent, should be equally effective. Therefore a study of the action of this drug on a condition of shock seemed justified, particularly in view of its usefulness in human medicine.

Hexamethonium salts (C_6) block the sympathetic and parasympathetic ganglionic synapses. This causes a relaxation of the vascular tree and results in a fall in blood pressure to a hypotensive level. There is no evidence for any direct action on blood vessels. The pressure response to adrenaline is enhanced owing to lack of autonomic regulation (13).

To investigate the effects of C_6 in experimental shock the application of tourniquets to both hindlegs of rats was chosen as a suitable method. This method has been studied frequently and is reported to give reproducible results (1, 7, 9, 15).

Manuscript received in original form February 22, 1955, and as revised, June 15, 1955.
Contribution from the Department of Pharmacology, University of Western Ontario, London, Ontario.

Methods

White male rats were used, weighing from 200 to 360 gm. The rats were fed a standard diet with water ad libitum until the beginning of the experiment. After anesthesia with intraperitoneal sodium pentobarbitone (40–50 mgm./kgm. of body weight) arterial occlusion was produced in both hindlegs with tourniquets consisting of rubber bands with attached strings. The tourniquets were left in place for $4\frac{1}{4}$ to $4\frac{1}{2}$ hr. and light anesthesia was maintained during this time by additional small doses of sodium pentobarbitone, if necessary, in order to prevent the animal injuring its hindlegs. No anesthesia was given after the release of the tourniquets and food and water were withheld for the following 24 hr. and then offered ad libitum to the surviving animals. A rat living 48 hr. after the tourniquets were released was regarded as a survivor.

Hemoglobin (Hb) was used as a measure of the hemoconcentration. For each determination 0.02 cc. of blood was used. Several estimations could thus be made without materially affecting the blood volume of the animal. The blood was obtained by cutting off the tip of the tail and having the tail hang down for a short time. In this way the necessary blood was obtained easily; as the degree of shock advanced a gentle milking of the tail, which did not affect the results, was often necessary. As no significant changes were observed during the period when the tourniquets were in place, the preshock Hb level was determined about 30 min. before the release of the tourniquets. All determinations were made in duplicate, using the cyanhemoglobin method (8) and a Beckman spectrophotometer.

In another group of rats, blood pressure was recorded from the carotid artery with a mercury manometer.

In order to obtain a measure of the amount of edema in the hindlegs, the fluid content of muscle tissue was determined (4) in a third group of rats. This was done by dissecting out pieces of muscle tissue from the same region of the hindlimbs in normal rats, and treated and untreated tourniquet rats. These pieces were dried for 18 hr. in an oven at 100° C. and the fluid content was calculated from the wet and dry weights and is expressed as per cent of the wet weight.

Hexamethonium bromide ("Vegolysen" Poulenc) was given intraperitoneally in a dosage of 15 mgm./kgm. body weight. The drug was used in an aqueous solution containing 10 mgm./cc. This dosage was well tolerated by control rats, and caused a period of hypotension of about 90 min.

Results

In the control group, three out of 22 rats (14%) survived 48 hr. Out of 28 rats treated with C_6 five minutes before the removal of the tourniquets, 20 (or 72%) survived. When C_6 was injected two hours after the tourniquets were released, the survival rate dropped to 43% and treatment after four hours did not appear to improve survival (Table I). No significant difference

TABLE I

SURVIVAL RATE

Procedure	No. of rats	Surviving 48 hr.		Significance of difference, P
		No.	%	
Controls	22	3	14	
All rats treated with C_6 at time of tourniquet release	28	20	72	$<.01$
Treated with single dose C_6 2 hr. after tourniquet release	7	3	43	$<.05$
Treated with two equal doses of C_6 4 and 8 hr. after tourniquet release	6	1	17	$>.05$
Treated with single dose C_6 at time of tourniquet release	16	11	69	$>.05$
Treated with C_6 at time of tourniquet release and equal doses 4 and 8 hr. after	12	9	75	

in survival was seen between rats which received one injection before tourniquet release and those which had two additional injections four and eight hours later.

Release of the tourniquets caused a fairly rapid fall in blood pressure in untreated rats, which was soon followed by a rise to a level somewhat below the original values. Thereafter the blood pressure fell steadily. The time relation of these events varied moderately from rat to rat. Following the injection of C_6 the blood pressure fell within two to three minutes to a level between 90 and 60 mm. Hg. Subsequent removal of the tourniquets did not cause any change in blood pressure (Fig. 1). During the remainder of the

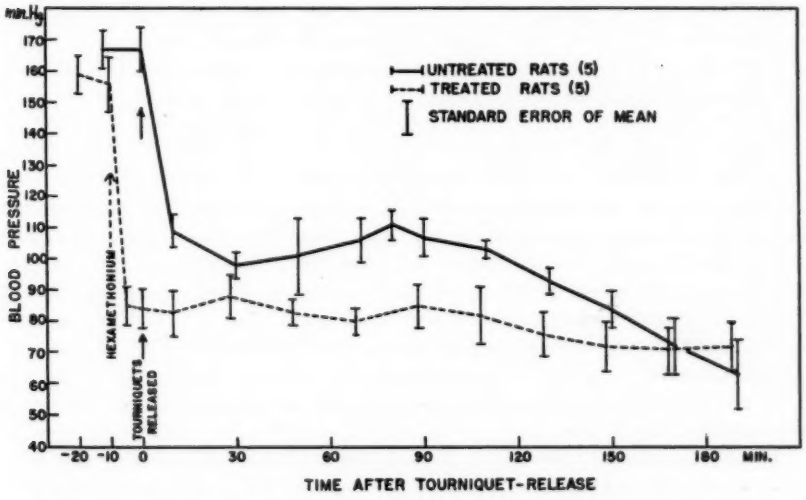


FIG. 1. Blood pressure in untreated and hexamethonium-treated tourniquet rats.

observation period only a very gradual decline in blood pressure was seen. The five treated rats appeared to be in good condition at the end of four hours of blood pressure recording, while only two out of five untreated rats survived this four-hour period. When C_6 was injected two hours after tourniquet release the extent of blood pressure fall was less and occurred more gradually and the blood pressure declined slowly during the following two hours.

The Hb level in seven untreated rats was 38.6 ± 3.0% (S.E.M.) above the preshock level eight hours after the tourniquets were released. In eight rats treated with C_6 before the tourniquets were removed, the increase in Hb was significantly less (27.9 ± 2.2% $P = <.01$).

Determination of the Hb level at intervals provided a pattern of hemoconcentration (Table II). In the untreated group the greatest rise in Hb was seen during the first two hours after tourniquet release. The main part of this increase occurred during the first hour. Subsequently a rather steady rate of increase was observed in the following two-hour intervals. Hexamethonium-treated rats, however, showed significantly less hemoconcentration during the first two hours, particularly during the first hour after tourniquet release. A decrease in the rate of hemoconcentration was observed in the following two-hourly intervals.

TABLE II

HEMOCONCENTRATION—INCREASE IN HEMOGLOBIN PER TIME INTERVAL IN GM.%; 0 DENOTES THE TIME OF RELEASE OF THE TOURNIQUETS

Hours	Controls		Treated with C_6		Significance of difference, P
	No. of rats	Increase in Hb, gm.% ± S.E.M.	No. of rats	Increase in Hb, gm.% ± S.E.M.	
0–1	13	1.85 ± 0.19	14	0.68 ± 0.15	<.01
0–2	22	2.43 ± 0.19	14	1.58 ± 0.17	<.01
2–4	15	1.25 ± 0.15	14	1.02 ± 0.11	>.2
4–6	6	1.28 ± 0.27	8	0.40 ± 0.17	<.01
6–8	7	1.09 ± 0.12	8	0.12 ± 0.11	<.01

When C_6 was given two hours after tourniquet release the progress of hemoconcentration was temporarily arrested. The Hb level remained unchanged in seven rats (−0.32 ± 0.28 gm.%) during the two hours following the injection, while the Hb level increased in the control group by 1.25 ± 0.15 gm.% and in the treated group by 1.02 ± 0.11 gm.% ($P = <.01$). An injection after four hours slowed the steady rate of hemoconcentration somewhat, but not significantly. In each case the injection of C_6 was followed by a visibly improved peripheral circulation, as indicated by the color of the skin and mucous membranes.

The fluid content of muscle tissue of the injured hindlimbs was significantly increased in the tourniquet rats. No difference could be demonstrated between untreated rats dying 20–24 hr. after tourniquet release and treated rats

TABLE III

FLUID CONTENT OF MUSCLE TISSUE OF HINDLEGS IN TOURNIQUET RATS 24 HR. AFTER TOURNIQUET RELEASE

Procedure	No. of determinations	Hindlegs, gm. of fluid per 100 gm. of muscle tissue (± S.E.M.)
Normal rats	5	75.2 ± 0.14
Tourniquet experiments untreated	6	82.2 ± 0.46
Tourniquet experiments treated with C_6 before and 4 hr. after release of the tourniquets	10	83.1 ± 0.30

sacrificed after 24 hr. (Table III). The most obvious swelling of the hindlegs occurred during the first two hours after the release of the tourniquets; no striking difference was seen in this respect between treated and untreated animals.

Discussion

In agreement with results obtained with other autonomic blocking agents in experimental shock (2, 6, 14, 17) the incidence of fatal tourniquet shock was significantly reduced by C_6 given at the time of tourniquet release. The success of later treatment with C_6 seemed to depend on the time which had elapsed since the release of the arterial occlusion: after two hours some effect was still evident but none after four hours. This suggests that a ganglionic blockade interferes with the initiation of the shock syndrome but becomes ineffective after shock has been in progress for a certain time.

It was shown above that the blood pressure response to the restoration of circulation through the hindlegs was significantly altered by C_6. In untreated rats the removal of the tourniquets was followed by a marked fall in blood pressure (3). After treatment of the animals with C_6 the blood pressure did not fall further on release of the tourniquets. The cause of this initial fall in blood pressure in the untreated rats was thought to be due to either a reduction of the blood volume due to local fluid loss or pooling of blood in the hindlimbs, or the liberation of some toxic substance which causes a generalized vasodilatation. With these factors a further fall in blood pressure should be expected after the release of the tourniquets in C_6-treated rats, since Moe *et al.* (10) and Paton and Zaimis (12) have shown that animals with a ganglionic block are very sensitive to a reduction of their blood volume and react to vasodilator substances with a fall in pressure in spite of the already existing hypotension. The lack of a blood pressure response to tourniquet release could therefore indicate that the mechanism causing the initial fall in blood pressure is mediated by the autonomic nervous system, i.e. the factor which initiates this response should act cephalad to the autonomic ganglia.

During the following period, which according to Chambers *et al.* (3) is characterized by vasoconstriction, a moderate rise and subsequently a gradual

fall in blood pressure was seen in the untreated rats. These changes were absent in the treated rats, presumably since vasoconstriction was inhibited by the ganglionic block.

The marked increase in hemoglobin during the first hour after tourniquet release was significantly reduced by treatment with C_6. This increase has been partly attributed to a contraction of the spleen (15), a mechanism which would be inhibited by a ganglionic block.

No difference in local fluid loss could be demonstrated between treated and untreated rats. If loss of fluid from the circulation into the damaged limbs would be the only cause of hemoconcentration, a close relation between the hemoconcentration and the local fluid loss should exist. It has been shown that fluid accumulates rapidly at first in damaged tissues but thereafter the rate of edema formation becomes progressively slower (9, 10). The observed pattern of hemoconcentration in treated animals appears to have a similar tendency in contrast to a steady rise in hemoglobin in the untreated rats. In the latter group other factors appear to contribute to the hemoconcentration, presumably a deterioration of the peripheral circulation; this was also indicated by the significant slowing of the rate of hemoconcentration following the improvement of the peripheral circulation when C_6 was given two hours after the release of the tourniquets.

Acknowledgments

The author is indebted to Prof. R. A. Waud, Head of the Department of Pharmacology, for his guidance and advice. The technical assistance by Mr. J. Boyd and Mr. R. Hunt is gratefully acknowledged. Vegolysen was provided by Poulenc Limited, Montreal.

References

1. Allan, F. M. Am. J. Surg. 60 : 335. 1943.
2. Baez, S., Zweifach, B. W., and Shorr, E. Federation Proc. 11 : 7. 1952.
3. Chambers, R., Zweifach, B. W., and Loewenstein, B. E. Ann. Surg. 120 : 791. 1944.
4. Fox, C. L., Jr. and Keston, A. S. Surg. Gynecol. Obstet. 80 : 561. 1945.
5. Freeman, N. E., Schaffer, S. A., Schechter, A. E., and Holling, H. E. J. Clin. Invest. 17 : 359. 1938.
6. Glasser, O. and Page, I. H. Am. J. Physiol. 154 : 297. 1948.
7. Hechter, O., Bergman, H. C., Sapirstein, L., Fish, R., Feigen, G., and Prinzmetal, M. Am. Heart J. 29 : 499. 1945.
8. King, E. J. and Gilchrist, M. Lancet, 253 : 201. 1947.
9. Koletzki, S. and Gustafson, G. E. J. Clin Invest. 24 : 839. 1945.
10. Moe, G. K., Rennick, B. R., Capo, L. R., and Marschall, M. R. Am. J. Physiol. 155 : 158. 1949.
11. Nickerson, J. L. Am. J. Physiol. 144 : 429. 1945.
12. Paton, W. D. M. and Zaimis, E. D. Brit. J. Pharmacol. 6 : 155. 1951.
13. Paton, W. D. M. and Zaimis, E. D. Pharmacol. Rev. 4 : 219. 1952.
14. Remington, J. W., Hamilton, W. F., Boyd, C. H., Jr., Hamilton, W. F., Jr., and Caddell, H. M. Am. J. Physiol. 161 : 116. 1950.
15. Shipley, E. G., Meyer, R. K., and McShan, W. H. Proc. Soc. Exptl. Biol. Med. 60 : 340. 1945.
16. Wiggers, C. J. Physiology of shock. The Commonwealth Fund, New York. 1950.
17. Wiggers, H. C., Ingraham, R. C., Roemhild, F., and Goldberg, H. Am. J. Physiol. 153 : 511. 1948.

ON RAT SERUM AMYLASE

III. THE CONTRIBUTION BY VARIOUS TISSUES TO SERUM AMYLASE ACTIVITY[1]

By G. Stuart Wiberg and Jules Tuba

Abstract

The contribution of various tissues to the level of rat serum amylase has been investigated. The pancreas appeared to furnish significant amounts of the enzyme to serum. This was suggested by parallel decreases in pancreatic and serum amylase activity associated with fasting, partial pancreatectomy, and parenteral administration of ethionine. However, increased levels of pancreatic amylase, produced by high dietary concentrations of starch and sucrose, were not paralleled in the serum. Major pancreatectomy indicated that extrapancreatic sources contribute to serum amylolytic activity. Fasting, partial pancreatectomy, and ethionine injection were followed by decreased amylase levels in serum and intestinal mucosa, and suggested the mucosa as a possible source of the serum enzyme. Depot fat contained appreciable amounts of amylase and is considered as a contributor to serum. This possibility is indicated by the highly significant increase produced in serum and depot fat amylase levels by a high dietary concentration of fat. Moreover a highly significant correlation was found between serum and depot fat amylase levels. It was noted that the high fat diet stimulated amylolytic activity of intestinal mucosa without affecting pancreatic amylase levels. The parotid glands had the highest tissue amylase levels but did not appear to contribute to the serum.

Introduction

Amylase is one of the enzymes found in serum that appear to have no catalytic function therein and the origin of which is usually a matter of conjecture rather than an established fact. The level of serum amylase is known to be elevated in acute pancreatitis and in paratoditis, but these increases reflect pathological states and there is no assurance that the organs involved make a comparable contribution in the normal animal. Somogyi (15) reviewed the literature up to 1941 and concluded that the pancreas, the liver, the parotid glands, or the intestine had no influence on serum amylase levels under physiological conditions. Wiberg, Little, and Tuba (17) found that partial ablation of the pancreas resulted in a highly significant decline in serum amylase levels in the rat, and that this decreased activity of the enzyme persisted up to 332 days postoperatively. They noted that partial hepatectomy did not affect serum amylase. Roe, Smith, and Treadwell (14), however, reported that total pancreatectomy in rats left the levels of the serum enzyme unchanged.

A further attempt by us to locate the sources of amylase in rat serum involved studies of the enzyme in various tissues. It was necessary first to establish the normal values in the tissues. Subsequently, rats were subjected to experimental conditions known to affect the level of the enzyme in the serum. Parallel changes in amylase activity in any tissue were considered

[1] *Manuscript received June 6, 1955.*

Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alberta, with financial assistance from the National Research Council, Ottawa, Canada.

by us to be presumptive evidence that such a tissue contributes some of the enzyme normally found in the serum. The experimental factors reported in this paper are: starvation (16, 17), pancreatectomy (17), diet (18), and intraperitoneal injection of DL-ethionine.

Carlson and Luckhardt examined the amylase levels of various body fluids (3). In general, they found amylase activities to be in the following decreasing order: serum, thoracic lymph, neck and limb lymph, pericardial fluid, and cerebrospinal fluid. Hirata (10) made a similar investigation of tissues in man and the dog, cat, rabbit, guinea pig, hen, fish, bullfrog, and rat, and reported enzyme activity in the following descending order: pancreas, serum, liver, kidney, muscle, and spleen. Subsequently, the presence of amylolytic activity has been reported in the adipose tissue of the dog, rat, rabbit, mouse, and man (4, 9, 12), and also in the lung of several species (13).

Some more recent studies of the distribution of tissue amylase activity have also been made. Yamagata (19) observed the following decreasing order of activity of the enzyme in the rabbit: pancreas, kidney, muscle, spleen, and liver. Okamoto (13) made an exhaustive study that included the rabbit, rat, guinea pig, bullfrog, chicken, and pigeon, and found the activity of the enzyme in decreasing order as follows: kidney, liver, lung, skin, muscle, and brain. He found that the rat and guinea pig possessed the highest tissue enzyme activity of the animals investigated. Roe *et al.* (14) placed the amylase activity in the following descending order for the rat: pancreas, liver, duodenum, jejunum, kidney, heart, spleen, and muscle.

Methods

After the animals were killed, the various organs were removed as quickly as possible and chilled. The tissue was weighed and then homogenized in a Potter–Elvehjem glass homogenizer with ice-cold 0.9% sodium chloride. The whole homogenates were used for amylase estimation. In general homogenates were diluted to the same order of activity as serum amylase. Sodium chloride (0.9%) served as the diluent.

The method employed for tissue amylase assay made use of the starch–iodide technique described for the estimation of serum amylase activity (16). The pH optimum was determined and corresponded to that for serum amylase (7.0) for all tissues investigated. The unit of amylase activity is that amount of enzyme sufficient to hydrolyze 1 gm. of starch in one hour at 37° C. at pH 7.0. Not more than 50% of the substrate should be hydrolyzed in the one-hour digestion period. Results are expressed as units of amylase activity per 100 gm. of wet tissue or per 100 ml. of serum.

Results

Tissue Levels of Amylase in Normal Rats

The amylase level of different tissues was measured in a group of 10 adult male rats. The results of this experiment are to be found in Table I. Three tissues, viz. the parotid glands, pancreas, and the intestinal mucosa, possess

very high levels of amylase activity in comparison with the serum. The parotid level is approximately twice that of the pancreas. No mention of this extremely high value has been found in the literature. The lungs, depot fat (perirenal), and the kidney also possessed considerable amylolytic activity. The enzyme level of the liver was somewhat lower and is probably due in large part to residual blood (15, 17). The testes and skeletal muscle showed slight amylolytic activity while the spleen, heart, and brain were completely devoid of demonstrable amylase action.

TABLE I

AMYLOLYTIC ACTIVITY OF RAT TISSUES

Tissue	Amylase activity, units/100 gm. wet tissue*	Range
Parotid glands	107,400 ± 25,000	77,000 – 151,000
Pancreas	54,700 ± 3850	30,000 – 80,000
Intestinal mucosa	428 ± 195	300 – 800
Serum	61.6 ± 3.4	57 – 62
Lung	37.7 ± 2.4	13 – 87
Depot fat (perirenal)	30.0 ± 2.0	13 – 40
Kidney	21.1 ± 2.3	16 – 27
Liver	13.1 ± 1.8	10 – 15
Testicle	4.5 ± 0.5	0 – 9.7
Muscle (thigh)	2.7 ± 0.2	0 – 5.9
Spleen	Nil	Nil
Heart	Nil	Nil
Brain	Nil	Nil

* *Each recorded value is the mean for 10 rats (plus or minus the standard error of the mean).*

Effects of Starvation and Pancreatectomy

The level of amylase activity was studied in rats which had been starved for seven days and in a group of animals which were killed 332 days after removal of 80–90% of the pancreas. In addition to the serum, the enzyme levels in the parotid glands, residual pancreatic tissue, and the intestinal mucosa were studied. These results are presented in Table II. For purposes of comparison the normal values recorded above in Table I are also included.

TABLE II

TISSUE AMYLASE LEVELS IN STARVED AND PANCREATECTOMIZED RATS

Tissue	Normal rats	Animals fasted seven days	Partially pancreatectomized rats
Serum	61.6 ± 3.4	21.9 ± 2.7	52.2 ± 3.8
Parotids	107,400 ± 25,000	164,800 ± 31,000	127,200 ± 29,500
Pancreas	54,700 ± 3850	10,050 ± 1750	
Intestinal mucosa	428 ± 95	14.3 ± 1.1	133 ± 42

Values shown are the mean of 10 animals (plus or minus the standard error of the mean). Amylase activity is per 100 gm. of wet tissue.

Starvation was accompanied by highly significant decreases in amylase of serum, pancreas, and intestinal mucosa ($P < 0.01$), but parotid amylase was not changed. It is possible that some of the decreased serum activity might be due to the drop in the level of the pancreatic enzyme. There is also the possibility that the disappearance of the depot fat during fasting resulted in some decrease in serum amylase levels. The amylase level of the intestinal mucosa is very much lower and this might be due to the decreased level of the pancreatic enzyme. It is not known whether the amylase of the intestinal mucosa is indigenous to the gut or whether it represents entrained pancreatic secretion. Every attempt was made to effect complete separation of pancreatic tissue from the intestine to avoid interference by the pancreatic enzyme with estimations of intestinal amylase activity.

Pancreatectomy resulted in highly significant decreases in both the serum and the mucosal amylase levels ($P < 0.01$ for each). Again the parotid gland amylase activity remained unaffected. The fall in the level of the mucosal amylase could have been due to the removal of the pancreas. Roe *et al.* (14) report a similar finding in the experiments with totally pancreatectomized rats. It was suggested by these workers that remaining amylase in the intestine could have been derived from the salivary glands.

Examination of Table II shows several other interesting features. The decrease in serum amylase is much greater in the starved rats than in the pancreatectomized animals although the former still retain considerable pancreatic amylase activity. Also the mucosal amylase level is lower in the starved animals. This would appear to indicate that while the pancreas does contribute to the maintenance of the serum amylase, a very large portion must be extrapancreatic in origin. Since the depot fat has disappeared in the fasted rats it might be that this tissue constitutes one source of the enzyme. Another possible source is the intestinal mucosa. The surgical technique employed in the pancreatectomies reported here did not permit the total removal of this organ hence there is no unequivocal evidence of the identity of mucosal amylase as an indigenous enzyme.

Effect of the Composition of the Diet

Since the serum amylase level in the rat is affected by alterations in the composition of the diet (18), the question arose whether these changes in serum amylase were accompanied by corresponding changes in tissue amylases. Four groups of six rats each were maintained on a high sucrose, high starch, high protein, or high fat diet for 28 days. The exact composition of these diets has previously been described (18). On the 28th day, the animals were sacrificed and the parotid glands, serum, pancreas, intestinal mucosa, and depot fat (perirenal) were analyzed for amylase content. The results are presented in Table III.

The effect of these diets on the serum amylase was reported and discussed in a previous paper of this series (18). Briefly, the high sucrose and high starch diets were without effect, while the high fat diet increased, and the high

TABLE III

EFFECT OF DIET ON TISSUE AMYLASE LEVELS

Diet	Serum	Parotid glands	Pancreas	Intestinal mucosa	Depot fat
Normal	59.7 ± 1.3	124,000 ± 24,500	51,300 ± 3450	437 ± 43	25.5 ± 1.5
High sucrose	60.3 ± 3.6	118,000 ± 27,800	77,600 ± 4620	653 ± 105	19.8 ± 1.8
High starch	62.8 ± 2.5	118,000 ± 29,200	90,000 ± 5210	824 ± 87	21.6 ± 2.2
High protein	51.7 ± 1.6	103,000 ± 26,300	52,300 ± 4380	324 ± 61	9.4 ± 0.08
High fat	66.5 ± 2.0	107,000 ± 19,600	57,000 ± 3760	848 ± 78	76.1 ± 4.7

Values, reported in units per 100 gm. wet tissue, represent the mean of six animals (plus or minus the standard error of the mean).

protein diet depressed serum amylase values. The constancy of the parotid gland amylase levels was again noted. The pancreatic enzyme, however, showed a highly significant elevation with both the high sucrose and the high starch diets ($P < 0.01$ for each). This increased level of the pancreatic amylase has been reported previously for high starch diets by Grossman, Greengard, and Ivy (8) and by Hirata (10). Although the amylase activity of the pancreas was increased, there was no corresponding increase in the serum. The high protein and the high fat diet did not influence the level of the pancreatic enzyme.

The changes in amylase activity of the mucosa paralleled, in the main, those of the pancreas with highly significant increases ($P < 0.01$) for the high sucrose and high starch regimens. There was a significant decline ($P < 0.05$) on the high protein diet and a highly significant increase ($P < 0.01$) on the high fat diet.

The effect of these diets on depot fat amylase is probably the most interesting. The high starch and the high sucrose diets were without effect, but the high protein diet resulted in a highly significant decline and the high fat diet in a highly significant increase in enzyme activity ($P < 0.01$ for both). These changes paralleled similar changes in the serum amylase. A highly significant correlation coefficient ($r = 0.784$, $P < 0.01$) was obtained between the levels of the amylase activity in the serum and the depot fat for those groups existing on the stock diet and those on the high fat diet. It is quite possible, therefore, that one of the factors that affected the level of the serum might be the amylolytic activity of the adipose tissue. The actual role of amylase in depot fat is unknown, but Mirski (12) has found considerable glycogen reserves in rat depot fat.

The high protein diet caused highly significant decreases ($P < 0.01$) in amylase activity of both the serum and the depot fat. It will be recalled that starvation or reduced food intake brought about decreased serum amylase levels (16, 17). Since this will also cause the adipose tissue to disappear, it may be that this is part of the explanation for the decreased serum amylase level in inanition.

Effect of Ethionine

The destruction of acinar tissue in rat pancreas by repeated doses of ethionine has been reported by several investigators (2, 5, 6, 7). Bollag and Gallico (2) observed that this destruction was accompanied by marked decrease of pancreatic amylase, and that this decrease was proportional to the degree of acinar damage. Almeida and Grossman (1) found similar changes in cats, dogs, and monkeys. These workers noted a transient fall in the serum amylase of the dog after ethionine administration. This decrease was followed by a sharp rise coincident with the development of pancreatitis. Kroboth and Hallenbeck (11) also found that, in dogs, the continued administration of ethionine for two to three weeks produced a transiently depressed plasma amylase, then a transiently elevated enzyme level, and finally a persistently depressed plasma amylase.

A series of experiments was undertaken to evaluate the effect of intraperitoneal injections of ethionine on rat serum amylase. In the course of these experiments, it was noted that the ethionine produced a state of intense anorexia. This condition itself will result in significantly lowered levels of rat serum amylase (16, 17). In order to circumvent this situation, the experiment was redesigned and a control group with paired feeding was also included.

A group of six male adult rats, housed in individual cages, was fed a diet of ground Purina fox checkers and water ad libitum. After one week, intraperitoneal injections of 100 mgm. of ethionine in 4 ml. of water were instituted and continued for a period of 14 days. Blood for amylase estimation was collected from the tail, just before the initial injection, and again on the 7th and the 14th day of the injections. After the final injection, the animals were killed and the pancreas, parotid glands, and the first 20 cm. of gut were removed for amylase determination. Daily records of food consumption were

TABLE IV

ETHIONINE INJECTION AND SERUM AMYLASE ACTIVITY

Day of treatment	Serum amylase, units/100 ml.	Body weight, gm.	Food consumption, gm./day
Preinjection	62.0 ± 2.1	263 ± 6.5	20
	59.3 ± 0.9	270 ± 3.2	20
7th day	29.4 ± 3.2	232	2
	36.3 ± 1.7	242	2
14th day	14.1 ± 1.7	175 ± 9.3	0
	27.8 ± 4.3	196 ± 4.8	0

Upper row of figures refers to experimental animals receiving ethionine.
Lower row of figures refers to control animals receiving saline.
Values shown are for the mean of six animals (plus or minus the standard error of the mean).

kept during the experiment. A second group of six animals served as controls and a paired feeding regimen was followed. This group received 4 ml. of physiological saline in lieu of the ethionine solution, otherwise the same experimental conditions were maintained throughout the experiment.

The effect of the ethionine injection of rat serum amylase is given in Table IV. At the start of the experiment no significant difference existed between the levels of the experimental and the control animals. At the end of seven days, both groups showed highly significant decreases from the initial values which were more marked in the ethionine group (experimental, $t = 9.6$; control, $t = 12.1$; $P < 0.01$ for both). This trend continued and was even more noticeable at the end of the 14th day of the ethionine injection (experimental, $t = 17.7$; control, $t = 7.0$; $P < 0.01$ for both).

Insofar as the control group was concerned, the decline in serum amylase may be attributed to the reduced food intake, an effect noted above. However, in addition to this phenomenon, there was a highly significant difference between the experimental and the control group at both the 7th and the 14th day of treatment, with the ethionine-treated rats showing the lower level (7th day, $t = 3.21$; 14th day, $t = 4.97$; $P < 0.01$ for both). This difference is presumably brought about by some factor other than food. Consideration of Table V, which gives the results of the tissue amylase determinations, reveals one possible explanation.

TABLE V

CHANGES IN AMYLASE LEVELS AFTER ETHIONINE INJECTION

Tissue	Experimental	Control	Normal*
Serum	14.1 ± 1.7	27.8 ± 4.3	61.6
Pancreas	584 ± 220	41,460 ± 2770	54,700
Intestinal mucosa	20.6 ± 5.0	67.0 ± 28	428
Parotid glands	101,000 ± 28,000	107,600 ± 34,000	107,400

Values shown are the mean of six animals (plus or minus the standard error of the mean). Amylase activity is in units per 100 gm. of wet tissue.

** To facilitate comparison, the values from the normal group of animals reported in Table I are included.*

Treatment with ethionine has brought about highly significant decreases in both the pancreatic ($t = 4.63$, $P < 0.01$) and the intestinal mucosal ($t = 3.49$, $P < 0.01$) enzyme levels while that of the parotid glands remained unchanged. Since there is also a highly significant difference between the serum amylase activities in the experimental and control groups, it is possible that this difference might be due in part to changes in the enzyme level of the pancreas and the intestinal mucosa, provided that the amylase found in the mucosa is indigenous to the gut.

Discussion

Extended fasting and prolonged administration of ethionine have both resulted in depressed amylolytic activity of the pancreas and serum of adult male rats. In conjunction with our previous finding that pancreatectomy lowered the level of rat serum amylase (17), there seems to be ample evidence that the pancreas is a primary source of the serum enzyme. These studies also show that a large fraction of rat serum amylase has an extrapancreatic origin.

Two potential sources of this fraction of the enzyme are the depot fat and the intestinal mucosa. The disappearance of the adipose tissue in animals undergoing starvation or treatment with ethionine (actually another example of inanition) was accompanied by a marked diminution of serum amylase, much greater than could be explained by lowered pancreatic function alone. Furthermore, the high fat diet elevated both the serum and the depot fat amylases although values for the pancreas were unaltered. Similarly, the high protein diet depressed enzyme activity of both serum and depot fat although the pancreatic amylase level remained well within the normal limits.

Much the same argument prevails for the role of the intestinal mucosa. The depressed values following pancreatectomy, starvation, and ethionine treatment paralleled those of the serum. Similar trends were observed with the high fat and high protein diets, i.e., elevated levels in the first instance, and decreased values of serum and intestinal mucosal amylases in the second instance. Interestingly enough, only on the high fat diet did an elevated serum amylase accompany an elevated intestinal mucosal enzyme level.

It had been hoped, either through the removal of the pancreas or the destruction of the acinar tissue of that organ, to demonstrate an independent origin of the amylase of the intestinal mucosa. Unfortunately, total ablation of the pancreas could not be realized, and the parenteral administration of ethionine was accompanied by many changes other than the destruction of the pancreatic acini. It was not possible therefore to indicate that the amylase of the intestinal mucosa originated therein rather than from entrained pancreatic secretion. Consequently it should be remembered that any part ascribed to the gut in its contribution to the maintenance of rat serum amylase levels largely may be due to pancreatic secretion. Roe *et al.* (14) found amylolytic activity in the duodenum and jejunum of the rat following complete pancreatectomy. This they attributed to salivary amylase, which would mean that the saliva must have travelled through the stomach avoiding contact with either pepsin or hydrochloric acid, since these two agents rapidly inactivate this enzyme, at the pH encountered in the gastric juice.

The significantly elevated pancreatic and intestinal mucosa amylase levels (apart from the mucosal amylase in the high fat diet) were not reflected by alterations in serum enzyme activity.

The parotid gland amylase remained unaltered by all the various experimental conditions investigated. It would seem therefore that its contribution to the amylase level of rat serum is negligible.

References

1. ALMEIDA, A. L. de and GROSSMAN, M. I. Gastroenterology, 20 : 554. 1952.
2. BOLLAG, W. and GALLICO, E. Biochim. et Biophys. Acta, 9 : 193. 1952.
3. CARLSON, A. J. and LUCKHARDT, A. B. Am. J. Physiol. 23 : 148. 1908. *Cited from* Chem. Abstr. 4 : 2152. 1910.
4. CEDRANGOLO, F. Atti Accad. Nazl. Lincei, Classe sci. fis. mat. enat. 25 : 137. 1937. *Cited from* Chem. Abstr. 31 : 7896. 1937.
5. FARBER, E. and POPPER, H. Proc. Soc. Exptl. Biol. Med. 74 : 838. 1950.
6. FITZGERALD, P. J. and ALVIZOURI, M. Nature, 170 : 929. 1952.
7. GOLDBERG, R. C., CHAIKOFF, I. L., and DODGE, A. H. Proc. Soc. Exptl. Biol. Med. 74 : 869. 1950.
8. GROSSMAN, M. L., GREENGARD, H., and IVY, A. C. Am. J. Physiol. 138 : 676. 1942-3.
9. HAUSBERGER, F. X. Z. ges. exptl. Med. 102 : 169. 1937. *Cited from* Chem. Abstr. 32 : 7087. 1938.
10. HIRATA, G. Biochem. Z. 27 : 384. 1910. *Cited from* Chem. Abstr. 5 : 332. 1911.
11. KROBOTH, F. J. and HALLENBECK, G. A. Gastroenetrology, 27 : 743. 1954.
12. MIRSKI, A. Biochem. J. 36 : 232. 1942.
13. OKAMOTO, S. J. Biochem. (Japan), 37 : 269. 1950. *Cited from* Chem. Abstr. 45 : 3438 1951.
14. ROE, J. H., Jr., SMITH, B. W., and TREADWELL, C. R. Proc. Soc. Exptl. Biol. Med 87 : 79. 1954.
15. SOMOGYI, M. Arch. Internal Med. 67 : 665. 1941.
16. TUBA, J. and WIBERG, G. S. Can. J. Med. Sci. 31 : 377. 1953.
17. WIBERG, G. S., LITTLE, M. W., and TUBA, J. Am. J. Physiol. 33 : 53. 1954.
18. WIBERG, G. S. and TUBA, J. Can. J. Biochem. Physiol. 33 : 46. 1955.
19. YAMAGATA, S. Tôhoku J. Exptl. Med. 46 : 1. 1943. *Cited from* Chem. Abstr. 43 : 3505. 1949.

SPECTROPHOTOMETRIC ANALYSIS OF MIXED SOLUTES AND CRUDE ORGAN EXTRACTS[1]

By E. Annau

Abstract

A procedure is presented by which the spectrophotometric separation of two mixed solutes, absorbing in the ultraviolet region, became possible. By the extension of the method to crude organ extracts, absorption spectra characteristic in their main points for proteins could be obtained selectively from the composed spectra of the extracts. At the same time a method is described for the preparation of crude organ extracts showing an apparently maximal transparency.

Introduction

The use of ultraviolet spectrophotometric measurements for the optical differentiation of mixed solutes is limited by the fact that the solutes show a composed absorption spectrum which is characterized by all constituents.

In dealing with toxicological problems the necessity arose for the development of a procedure by which spectrophotometric separation of some constituents in crude tissue extracts could be obtained. This seems to have been achieved by a method based on the following observations: light passing through a sample composed of two different solutes will show the absorption spectrum of only one if (1) the absorption maxima of the two components are well separated from each other, and (2) the transmittance of the reference solution containing one of the components of the sample is adjusted to 100% transmittance through slit width operations.

The ultraviolet absorption spectra of various tissue extracts are closely similar and show a more or less S-shaped absorption band with a broad maximum between the wavelengths 250 and 270 mμ. This wide area of a homogenous and rather undifferentiated absorption band makes analytical spectrophotometry impracticable in this case.

It is a well known observation that among the different cell constituents of tissue preparations or extracts, only nucleic acids and proteins show defined absorption spectra, the pattern being characterized mainly by these two cell constituents. Since the absorption maxima of nucleic acids and proteins are at 260 and 280 mμ respectively, thus well separated from each other, it was suggested, according to the above observations, that the composite spectrum of tissue extracts might be reduced to their protein constituents only if the spectrum of the nucleic acids were eliminated from it optically.

At the same time attempts were made to prepare crude tissue extracts of maximal transparency for spectrophotometrical measurements.

The present communication deals with the results of these studies.

[1] *Manuscript received June 6, 1955.*
Contribution from Animal Pathology Division, Canada Department of Agriculture, Animal Diseases Research Institute, Hull, Quebec.

Materials and Methods

The preliminary experiments were carried out in aqueous solutions of the indicated concentrations of the following substances: 2×10^{-4} molar (M) solutions of hypoxanthine, xanthine, and uric acid, dissolved in distilled water by addition of sodium hydroxide; 4×10^{-5} M tryptophan solution, 0.03% solution of serum albumin, and 0.001% solution of commercial sodium ribonucleate (Schwartz Laboratories, New York 17).

The organ extracts were prepared from brains, liver, and thyroid glands of male albino mice, derived from the stock colony of this Institute and fed Master Fox Starter Breeder laboratory chow. The mice were bled through decapitation and the respective organs removed. Whole organs or organ pieces weighing approximately 200 mgm. were used for the extractions. In the case of the brain, including cerebrum, cerebellum, and stem, which on the average weighed 468 mgm., only one symmetrical half of the organ was used, while in the case of the thyroid gland the whole organ weighing approximately 200 mgm. served for the extraction. On the other hand, liver extracts were prepared from 200 mgm. pieces. Each organ or portion of organ was put in the center of a filter paper disk 9 mm. in diameter and was placed in a precooled Petri dish. H. Reeve Angel & Co., "made in Canada", filter paper was preferred for the experiments since it shows a well creeped surface and an open structure. The organs were crushed by a small pestle and rubbed carefully with a slight pressure into the filter paper on an area covering approximately 4 cm. in diameter. The remaining portion of the paper was cut off and discarded. The residuum containing the pressed tissue was replaced in the ice-cooled Petri dish, 10 ml. cold $M/1$ NaCl solution containing $M/15$ phosphate buffer of pH 7.00 was added and extracted for three hours at 5° C. in the cold room. The slightly opaque extract which contained a small amount of fine corpuscular elements was centrifuged for 25 min. at 18,000 r.p.m. at 0° C. in an International refrigerated centrifuge. The supernatant was practically transparent after having been diluted with distilled water in the following proportion: brain 1 to 10, liver or thyroid gland 1 to 20.

The optical measurements were carried out in the Beckman DU model spectrophotometer using standard 1 cm. silica cells with the selector switch in the position 1.0. Readings were made in the preliminary experiments at 5 mμ intervals, while in the case of the tissue extracts the intervals were reduced to 2 mμ in the region of maximal absorption. To obtain the highest possible precision the average of four parallel readings was taken with a permissible error of $\pm 0.8\%$. According to the nature of the experiment, the reference cell contained either distilled water or one of the components of the respective samples. Whenever selective absorption spectra of tissue extracts were determined the reference cell contained a 0.001% ribonucleate solution having the same salt concentration as the sample.

Results

Hypoxanthine, xanthine, and uric acid were chosen for the preliminary experiments since they show well distinguished absorption maxima at the wavelengths 250, 270, and 290 mμ respectively (1).

Fig. 1, Curve 2 gives the absorption spectrum of a 2×10^{-4} *M* hypoxanthine solution, and Curve 1 the composite spectrum of 2×10^{-4} *M* hypoxanthine and uric acid solutions mixed in equal amounts. In both instances distilled water was used as the reference solution. Curve 3 is the repetition of the latter experiment except that in the reference cell distilled water was replaced by a 2.4×10^{-4} *M* uric acid solution. It is quite obvious that in this case the composite absorption spectrum of the two components became reduced to that of hypoxanthine alone. Yet, it differs in two points from the spectrum of the pure hypoxanthine solution: it reveals a higher optical density and there is still present a residual absorption in the region of the uric acid maximum. These discrepancies however do not seem to be consistent and may be overcome using an approximate concentration of the reference solutes, as will be seen in the subsequent experiments.

Fig. 2 shows experiments in which a 2×10^{-4} *M* solution of xanthine and the mixed solutions of xanthine and hypoxanthine of the same molarity and in equal concentrations served for the selective spectrophotometric measurements. It may be seen in Curve 1 that the spectrum of the mixed solutes using distilled water as reference solution presents a broad absorption band between the wavelengths 250 and 260 mμ with a slight increment towards the longer wavelengths; employment of a 2.4×10^{-4} *M* solution of hypoxanthine

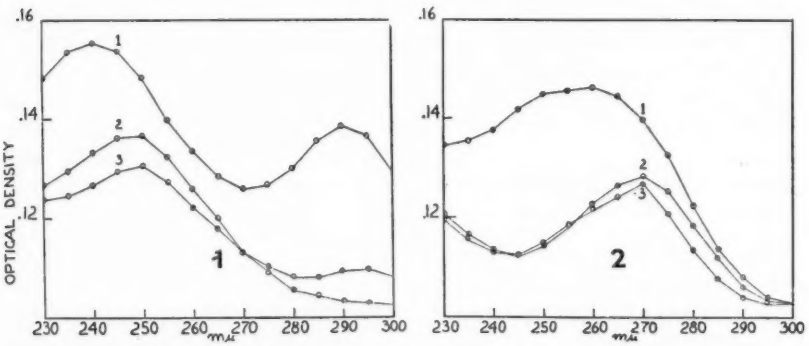


FIG. 1. Curve 1, sample: equal amounts of 2×10^{-4} *M* hypoxanthine and 2×10^{-4} *M* uric acid solutions; reference solution: dist. water. Curve 2, sample: 2×10^{-4} *M* hypoxanthine solution; reference solution: dist. water. Curve 3, sample: equal amounts of 2×10^{-4} *M* hypoxanthine and 2×10^{-4} *M* uric acid solutions; reference solutions: 2×10^{-4} *M* uric acid solution.

FIG. 2. Curve 1, sample: equal amounts of 2×10^{-4} *M* hypoxanthine and 2×10^{-4} *M* xanthine solutions; reference solutions: dist. water. Curve 2, sample: 2×10^{-4} *M* xanthine solution; reference solution: dist. water. Curve 3, sample: equal amounts 2×10^{-4} *M* hypoxanthine and 2×10^{-4} *M* xanthine solutions; reference solution: 2.4×10^{-4} *M* hypoxanthine solution.

instead of distilled water as the reference solution causes the broad absorption band of the mixed solutes to be replaced by the distinct spectrum of xanthine alone (Curve 3).

Since it was known that crude tissue extracts contain as main constituents both proteins and nucleic acids, and that the ultraviolet absorption spectra of proteins are due mainly to their aromatic amino acids, model experiments were carried out with a 4×10^{-5} *M* tryptophan solution and with a tryptophan solution of the same molarity supplemented with sodium ribonucleate in a concentration of 0.001%. All other conditions were the same as in the experiments described above. It may be seen from Fig. 3 that the absorption curve of the tryptophan–ribonucleate solution using distilled water as reference solution (Curve 1) shows a maximum at 265 mμ with a slight decrement towards 280 mμ, thereafter a steep decline of the curve occurs. It becomes also evident that the specific absorption curves both of ribonucleate and of tryptophan can be extracted optically from their composed absorption spectra by using alternatively either one of the components as reference solutes (Curves 2 and 3 and symbol).

It has been already mentioned that the selective absorption curves of hypoxanthine, as extracted from its composite spectrum with uric acid, did not have the same optical density as that of the pure hypoxanthine solution. In order to investigate this phenomenon more closely, experiments were carried out with samples containing 0.03% serum albumin and 0.001% sodium ribonucleate. Various concentrations of sodium ribonucleate served as reference solutions. As can be seen in Fig. 4 the optical density values of the selective protein spectra were related to the concentrations of the sodium

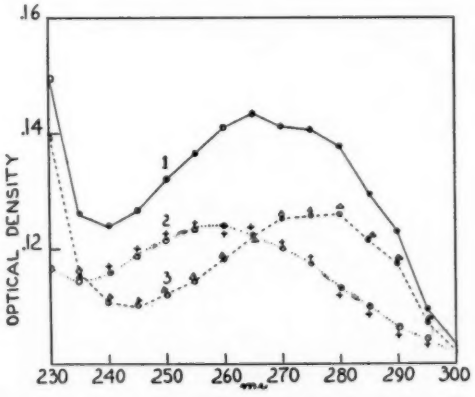


FIG. 3. Curve 1, sample: 4×10^{-5} *M* tryptophan solution containing 0.001% sodium ribonucleate; reference solution: dist. water. Curve 2, sample: 0.001% sodium ribonucleate solution; reference solution: dist. water. +, sample: 4×10^{-5} *M* tryptophan solution containing 0.001% sodium ribonucleate; reference solution: 4×10^{-5} *M* tryptophan solution. Curve 3, sample: 4×10^{-5} *M* tryptophan solution; reference solution: dist. water. Δ, sample: 4×10^{-5} *M* tryptophan solution containing 0.001% sodium ribonucleate; reference solution: 0.001% sodium ribonucleate.

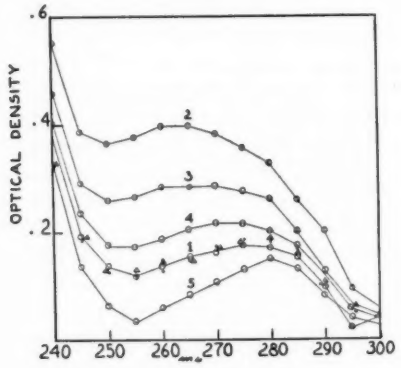


FIG. 4. Curve 1, sample: 0.03% serum albumin solution in dist. water; reference solution: dist. water. Curve 2, sample: 0.03% serum albumin solution containing 0.001% sodium ribonucleate; reference solution: dist. water. The samples of the succeeding curves are of the same composition as above, on the other hand the ribonucleate concentration of the reference solutions varies as follows: Curve 3, 0.0004%; Curve 4, 0.0008%; Curve 5, 0.0016%. The symbols ○ and △ correspond to reference solutions containing 0.001 and 0.0012% sodium ribonucleate respectively.

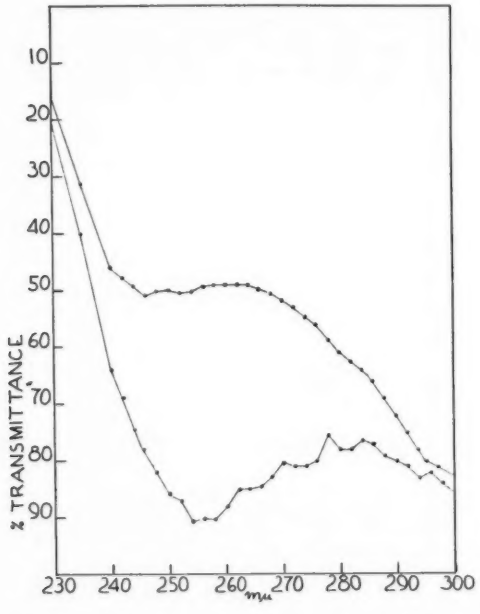


FIG. 5. Upper curve, sample: mouse brain extract diluted 1 part in 10 with dist. water; reference solutions: dist. water. Lower curve, the same sample as above; reference solution: 0.001% sodium ribonucleate solution.

ribonucleate of the reference solution. Optimal curves have been obtained when the concentration of sodium ribonucleate of the reference solution was closest to that of the sample or exceeded it by a small amount.

To produce selective protein spectra in crude tissue extracts a 0.001% sodium ribonucleate solution was used exclusively. Fig. 5 gives the absorption spectra of a mouse brain extract which is presented for better display in terms of per cent transmittance. In the experiment represented by the upper curve the reference solution consisted of an accordingly diluted pure extraction fluid. The curve is of the type we commonly found in all tissue extracts examined so far. It shows an approximately S-shaped broad absorption band with maxima between 250 and 270 mμ. It does not reveal any particular details and seems impracticable for analyses. The lower curve shows the absorption spectrum of the same extract, except that in this case a 0.001% ribonucleate solution was used as a reference solution. The difference between the two curves is apparent. No appreciable absorption is recognizable at 260 mμ, which is characteristic for nucleic acids. However, an absorption curve becomes evident beyond this point rising through different peaks to a maximum around 280 mμ which is characteristic for proteins.

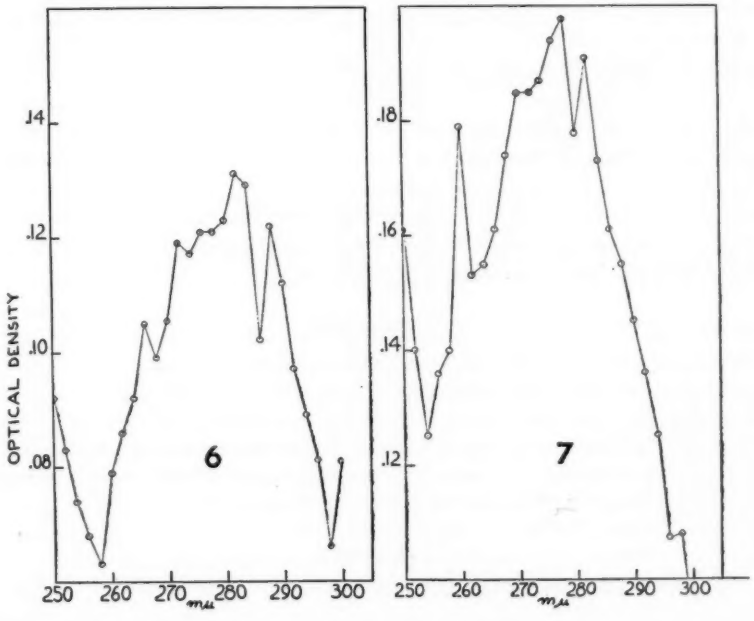


FIG. 6. Sample: mouse brain extract diluted 1 part in 10 with dist. water; reference solution: 0.001% sodium ribonucleate solution.

FIG. 7. Sample: mouse liver extract diluted 1 part in 20 with dist. water; reference solution: 0.001% sodium ribonucleate solution.

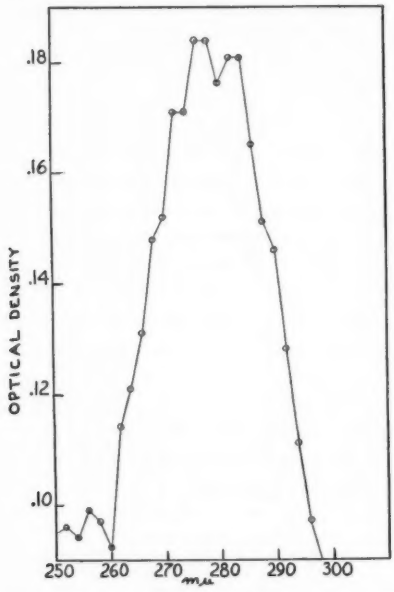


FIG. 8. Sample: mouse thyroid extract diluted 1 part in 20 with dist. water; reference solution: 0.001% sodium ribonucleate solution.

Figs. 6, 7, and 8 show the selective absorption spectra of crude extracts prepared from brain, liver, and thyroid gland tissues using in all instances 0.001% sodium ribonucleate as reference solution. All values are plotted in terms of optical densities. It can be seen from the figures that the extract of each organ was characterized by a particular absorption pattern, the least complicated one being that of the thyroid gland.

Conclusion

An optical procedure has been presented by which the composed ultraviolet spectrum of two different solutes could be reduced to the spectrum of one. Its applicability has been shown for systems consisting of two components with clearly distinct and widely separated absorption maxima, and possibly also for tissue extracts. A question that still has to be answered is whether this procedure can be extended to other more complicated systems.

Reference

1. KALCKAR, H. M. J. Biol. Chem. 167 : 429. 1947.

ULTRAVIOLET LIGHT ABSORPTION IN CRUDE ORGAN EXTRACTS FROM NORMAL MICE AND FROM MICE UNDER THE STRESS OF SOME TOXIC AGENTS[1]

By E. Annau

Abstract

Ultraviolet spectrophotometric studies of brain and liver extracts of normal mice and of mice suffering a progressive aldrin intoxication or an acute cocaine poisoning were described. Using an optical method developed in this Institute and presented in a previous communication, tentative protein spectra characteristic for each individual organ could be obtained. Essential differences could be registered in the absorption spectra from animals poisoned with aldrin or cocaine as compared to the spectra of controls.

Introduction

A spectrophotometric procedure was described in a previous communication by which the composed absorption spectrum of crude tissue extracts could be reduced selectively to a single spectrum characteristic for proteins (1). It was shown at the same time that extracts derived from different organs of normal mice, such as brain, liver, and thyroid gland, display apparently distinct absorption spectra. In the present study attempts were made to show that the selective absorption spectra of the same organ in different animals exhibit well recognizable diversities. It appears further that the selective absorption spectra of brain and liver extracts underwent alteration under the noxious stress of some drugs as for instance aldrin and cocaine.

Materials and Methods

Male albino mice of 25–30 gm. were used in this experiment, derived from the stock colony of this Institute and fed on Master Fox Breeder Starter laboratory chow.

To induce a progressive hyperexcitability of the central nervous system in the mice, aldrin in the form of corn oil solution was mixed to the ground basic diet in ratios of 30 or 50 parts per million. Acute intoxication of the central nervous system on the other hand was produced by the subcutaneous injection of 8 mgm. of pure cocaine in the form of a 4% corn oil solution. In this latter case hyperexcitability appeared usually within 10 min. after the injection, followed by repeated convulsions which lasted in general one or two hours. Generally, the animals were killed 45 min. after the onset of the first convulsion, exhibiting grave symptoms of cocaine intoxication.

The mice were bled by decapitation and their brain and liver instantly removed. Procedures for the preparation of organ extracts were identical with those already described (1), and thus may be omitted here.

[1] *Manuscript received June 6, 1955.*
Contribution from Animal Pathology Division, Canada Department of Agriculture, Animal Diseases Research Institute, Hull, Quebec.

Optical measurements were made in the Beckman DU model spectrophotometer. Readings were carried out using standard 1 cm. silica cells with the selector switch in the position 1.0. For the spectrophotometric measurements brain and liver extracts were diluted with distilled water in a ratio 1 to 10 and 1 to 20 respectively. A 0.001% solution of commercial sodium ribonucleate (Schwartz Laboratories, New York 17) was employed as reference solution having the same salt and buffer concentration as the sample. Optical measurements were made by taking 2 mμ readings and are given either in optical densities or in terms of per cent transmittance.

Results

The results are presented in graphs. Fig. 6 of a previous paper (1) shows the selective absorption spectrum of a brain extract from a control mouse. Fig. 1 (below) represents the spectrum of an experimental mouse fed on a diet containing 30 p.p.m. aldrin for a period of 32 days. As has been shown in previous reports (2, 3) the poisoned mouse exhibited a high degree of hyperexcitability and its liver was considerably enlarged. The differences between the two absorption curves are easily recognizable. The curve of the control animal is well differentiated showing several peaks and a maximum at the region of 280 mμ, while that of the experimental animal is more uniform and no characteristic peaks are apparent.

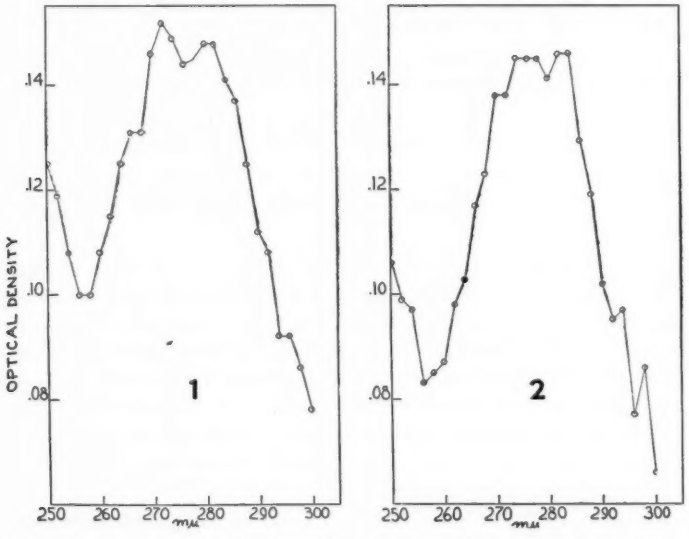


FIG. 1. Sample: mouse brain extract (dilution 1 to 10) of an experimental animal fed on a diet containing 30 p.p.m. aldrin for 32 days; reference solution: 0.001% sodium ribonucleate solution.

FIG. 2. Sample: mouse brain extract (dilution 1 to 10) of an experimental animal injected subcutaneously with 8 mgm. cocaine in form of corn oil solution, and killed 3 hr. after the onset of convulsions.

The absorption curve of a brain extract from an animal which had been exposed to an acute cocaine poisoning for two hours is given in Fig. 2. It can be seen that the curve of the cocaine intoxicated brain differs distinctly from that of a control mouse (Fig. 6 (Ref. 1)), while on the other hand it exhibits a strong similarity to the absorption curve of the sample from the aldrin treated animal (Fig. 1).

Fig. 3 represents the selective absorption spectrum of a normal mouse liver, and Fig. 4 that of an experimental animal fed a diet containing 50 p.p.m. aldrin. After a feeding period of 14 days the mouse revealed a well distinct hyperexcitability and its liver was enlarged. The tendency for a generalization of the absorption curve appears also in this case though it is not so apparent as in the curve from the brain since the absorption spectra of normal mouse livers are less complicated than those of the brain extracts.

To give a brief survey of more than fifty experiments Figs. 5–8 include several absorption curves of brain and liver extracts from control and experimental animals fed an aldrin diet in sublethal concentrations. Fig. 9 represents the experiments where cocaine was used as a toxic agent. For practical reasons all the curves are shown in terms of per cent transmittance but without numerical values. Consequently they have to be considered merely as absorption patterns. The measurements cover a spectral area between the wavelengths 250 and 300 mμ and are taken at 2 mμ intervals.

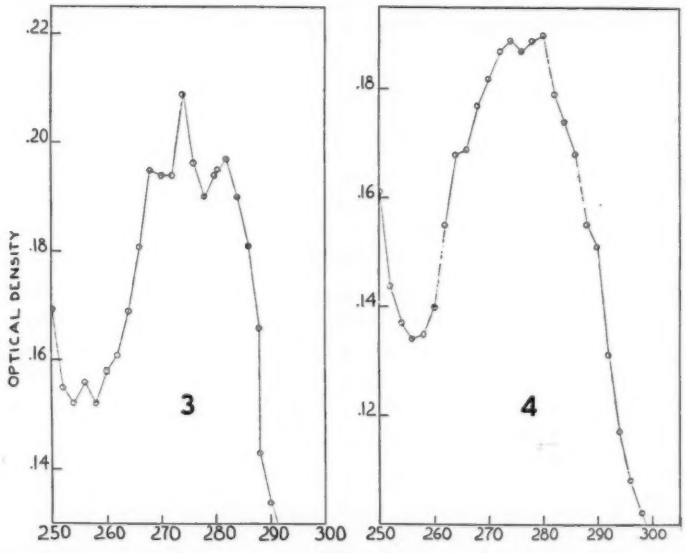
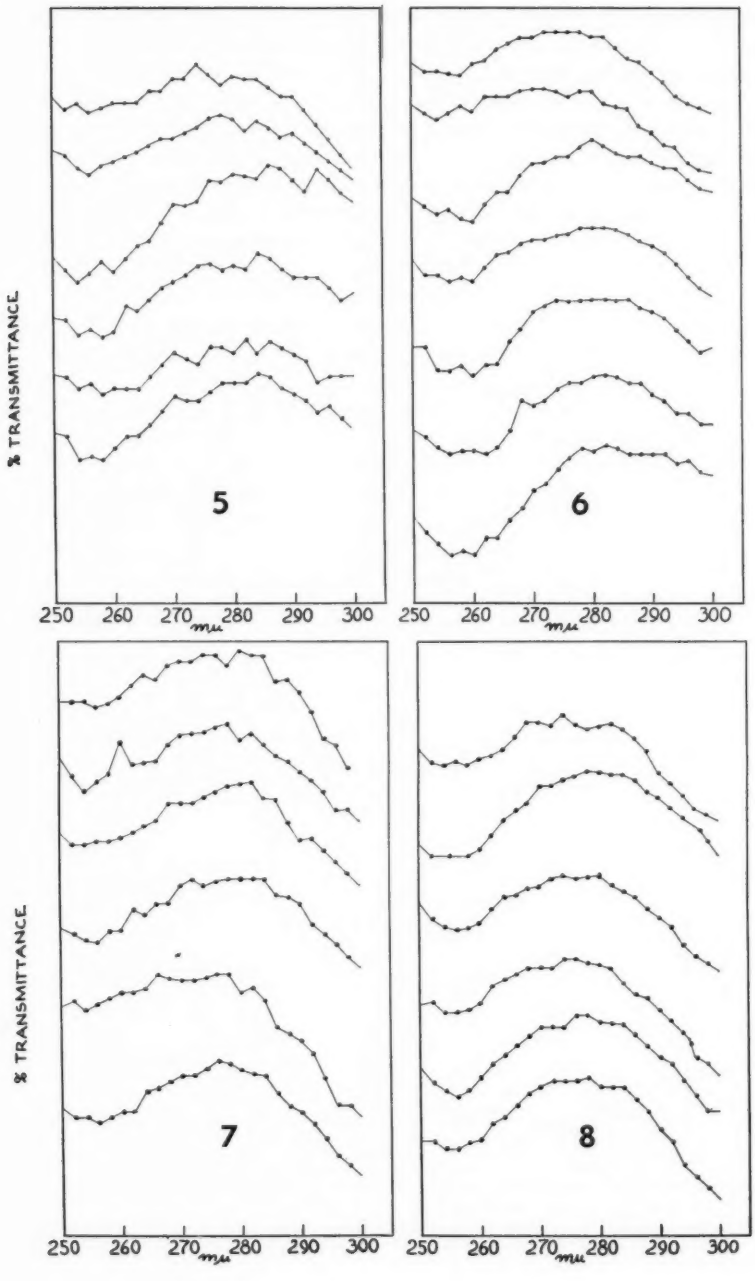


FIG. 3. Sample: mouse liver extract (dilution 1 to 10) of a normal animal, diluted 1 part in 20 with dist. water; reference solution: 0.001% sodium ribonucleate solution.

FIG. 4. Sample: mouse liver extract (dilution 1 to 20) of an experimental animal fed on a diet containing 50 p.p.m. aldrin for 14 days; reference solution: 0.001% sodium ribonucleate solution.


% TRANSMITTANCE
5
6
7
8
250 260 270 280 290 300
mμ

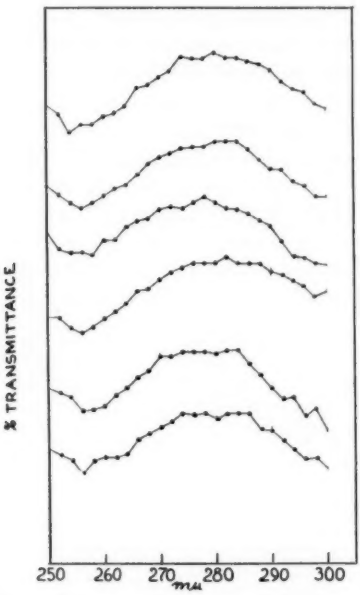


FIG. 9. Ultraviolet absorption curves of brain extracts from six mice following a subcutaneous injection of 8 mgm. cocaine in form of corn oil solution, expressed in per cent transmittance.

It can be seen from the patterns presented that a difference exists in the majority of the cases between the absorption curves of the organ extracts from normal and intoxicated animals. It is also apparent that the absorption patterns from the brain extracts of normal mice exhibit a somewhat more intricate fine structure compared with those of the liver extracts.

Discussion

From the point of view of spectrophotometry, crude organ extracts appear to be composed of two main constituents, nucleic acids and proteins. As it is well known both of these compounds have a sharply distinct absorption spectrum with a maximum at the wavelengths 260 and 280 mμ respectively. Yet their optical differentiation in crude extracts is impracticable since their

---

FIG. 5. Ultraviolet absorption curves of brain extracts from six normal mice expressed in per cent transmittance.

FIG. 6. Ultraviolet absorption curves of brain extracts from seven experimental mice suffering a progressive aldrin intoxication of different concentrations for various time intervals, expressed in per cent transmittance.

FIG. 7. Ultraviolet absorption curves of liver extracts from six normal mice, expressed in per cent transmittance.

FIG. 8. Ultraviolet absorption curves of liver extracts from six mice following a progressive aldrin intoxication of different concentrations for various time intervals, expressed in per cent transmittance.

spectra overlap into a broad absorption band of a rather undistinguishable character. This however can be altered fundamentally and a spectrum of the "protein" component produced if the light incident upon the sample is adjusted to 100% transmittance with regard to its nucleic acid content, as described in a previous communication (1). Yet the selective protein spectra of organ extracts as compared with the spectra of albumin solutions display essential dissimilarities. In contrast to the spectra of pure protein solutions they exhibit a particularly fine structure with a complexity of absorption peaks which seem to be characteristic not only for different organs but also for the same organs in single animals. The individual differences in the fine structure of single organs may be compared with the finger print regions in the infrared spectra of individual tissue preparations described by Schwartz and associates (5). In both instances however, it is impossible at least for the present time to give assignments to the different peaks along the absorption band. The suggestion that they might be related in some extent to enzymatic activities may be supported by the observations that they undergo alterations during exposure at room temperature or even disappear after dialysis. This hypothesis might be strengthened further by data from animals under the impact of certain toxic agents, as for instance aldrin or cocaine, according to which the absorption curves of brain extracts exhibit a definite simplification. A similar effect has been also reported by Schwartz and associates (5), who found in the majority of their experiments fundamental differences in the K values in brain sections from animals in insulin shock compared with the K values from normal rabbits. Recently Kovacs (4) published extensive studies on ultraviolet spectrophotometric measurements of cerebrospinal fluids derived from normal and pathological cases. According to his findings a great variety of pathological alterations in the central nervous system could be traced in the cerebrospinal fluid. He first described changes in the absorption curves occurring in the specimens after long standing at room temperature or after incubation at various temperatures and periods of time. However, still more experiments have to be carried out before any statement of general significance could be made.

References

1. ANNAU, E. Can. J. Biochem. Physiol. 33 : 826. 1955.
2. ANNAU, E., KONST, H., and PLUMMER, P. J. G. Can. J. Med. Sci. 30 : 463. 1952.
3. ANNAU, E. and KONST, H. Can. J. Med. Sci. 31 : 146. 1953.
4. KOVACS, E. Can. J. Biochem. Physiol. 32 : 526. 1954.
5. SCHWARTZ, H. P., RIGGS, H. E., GLICK, C., CAMERON, W., BEYER, E., JAFFE, B., and TROMBETTA, L. Proc. Soc. Exptl. Biol. Med. 76 : 267. 1951.

THE BINDING OF SULPHATASE BY RAT-LIVER PARTICLES AS COMPARED TO THAT OF ACID PHOSPHATASE[1]

By Renée Viala and R. Gianetto

Abstract

One of the sulphatases of rat liver, like acid phosphatase, is enclosed within cytoplasmic granules. The two enzymes are released from these granules in similar proportions by various treatments.

Introduction

It has been shown by de Duve *et al.* (5) and by Appelmans *et al.* (2) that the unspecific acid phosphatase of rat liver is bound to cytoplasmic granules which form a separate group, distinct from the cytochrome oxidase-bearing granules and from the glucose-6-phosphatase-containing microsomes.

When the morphological integrity of these granules is preserved, they show very little enzymic activity. However they are easily activated by a number of treatments which cause a simultaneous release of the enzyme in soluble form (1, 3, 4, 10).

Gianetto and de Duve (10) have reported recently that two other enzymes, β-glucuronidase and cathepsin, behave like acid phosphatase. Both enzymes are partly unreactive towards their respective added substrates, when in the bound state, and are simultaneously solubilized and fully activated by all the treatments which have been found to cause a similar release of acid phosphatase.

More recently de Duve *et al.* (6) have shown this distinct class of granules to contain two more hydrolases, ribonuclease and deoxyribonuclease. These two enzymes also share the cytological properties described for acid phosphatase, β-glucuronidase, and cathepsin.

The intracellular distribution of rat-liver sulphatase, assayed in the presence of 2-hydroxy-5-nitrophenylsulphate, reported by Roy (14), resembles that of unspecific acid phosphatase. Dodgson *et al.* (7), employing *p*-acetylphenylsulphate as substrate, found that the sulphatase of rat liver is localized mainly in the microsomes. Their results were confirmed in this laboratory, and the intracellular localization of rat-liver sulphatase, assayed in the presence of potassium *p*-acetylphenylsulphate, was shown to be identical to that of glucose-6-phosphatase (11), which was described by Hers *et al.* (12) as a typical microsomal enzyme.

Dodgson *et al.* (8), using 2-hydroxy-5-nitrophenylsulphate, have recently confirmed the findings of Roy (14) and have reported the existence of three sulphatases in rat liver. One of these sulphatases, the 2-hydroxy-5-nitrophenylsulphate-specific sulphatase, has an intracellular distribution resembling

[1] *Manuscript received May 19, 1955.*

Contribution from the Department of Biochemistry, Faculty of Medicine, University of Montreal, Montreal, Que. This paper is from a thesis presented by Renée Viala to the Faculty of Medicine in partial fulfillment of the requirements for the degree of Master of Science.

that of acid phosphatase. Furthermore it is activated by the same methods which cause complete liberation of acid phosphatase. Accordingly a comparative study of the similar release of this enzyme and acid phosphatase was made.

A close parallelism, both qualitative and quantitative, in the manner in which the two enzymes are released, has been revealed by these studies.

Methods

The method described by Appelmans and de Duve (1) was employed to isolate the once-washed mitochondrial fractions from the livers of Wistar male rats. The suspension medium was 0.25 *M* sucrose. The preparations were submitted to the treatment chosen to cause partial activation, following which the free enzyme activities were determined by an incubation of 10 min. at 37° C. in the presence of the substrate and sufficient sucrose to make the concentration of this sugar 0.25 *M* in the mixture. These conditions have been shown to maintain the integrity of the acid phosphatase-bearing granules (4) and permit a selective estimation of the percentage of free enzyme present in the incubation mixture.

Total activities were measured similarly on preparations previously diluted 10-fold with glass-distilled water and subsequently treated for a period of one minute in a cooled Waring blendor. The sulphatase activity of this preparation was immediately assayed, since after blendor treatment this enzyme undergoes slight denaturation on standing at 0° C.

Acid phosphatase assays were made in the presence of 0.05 *M* β-glycerophosphate adjusted to pH 5.5 with hydrochloric acid and buffered with 0.025 *M* acetate. The reaction was stopped with 10 ml. of 8% (w/v) trichloroacetic acid and the inorganic phosphate determined on the filtrate according to Fiske and Subbarow (9), using a Klett–Summerson photoelectric colorimeter. The total volume of the incubation mixture was 2 ml. and suitable blanks were always run.

Sulphatase activity tests were made using a slight modification of the method described by Roy (13). The incubation was carried out in a total volume of 2 ml., containing 0.0075 *M* 2-hydroxy-5-nitrophenylsulphate and 0.1 *M* acetate buffer pH 5.5. The reaction was stopped by the addition of 7.5 ml. of a 2% (w/v) phosphotungstic acid solution in 0.1 *N* hydrochloric acid. Denatured proteins were removed by filtration and the amount of 4-nitrocatechol set free was determined on a 3 ml. portion of the filtrate after the addition of 5 ml. of the alkaline quinol solution (13). The optical density of the red color developed was measured in the Klett–Summerson photoelectric colorimeter, using a green light filter (No. 52). Suitable controls were always run.

The amount of 4-nitrocatechol liberated by enzymic hydrolysis is proportional to the time of incubation but not to the amount of mitochondrial preparation used. Consequently the same amount of granules (1 ml.) was used in each experiment.

The dipotassium salt of 2-hydroxy-5-nitrophenylsulphate was synthesized according to the method of Roy (13). The product was recrystallized several times from water and yielded the theoretical amount of 4-nitrocatechol after acid hydrolysis.

Results

It is obvious from the results reported below that the 2-hydroxy-5-nitrophenylsulphate-specific sulphatase bears a striking resemblance to acid phosphatase in its behavior. Not only do their properties resemble each other qualitatively but the two enzymes can be released to the same extent from the special class of granules which contain them.

Osmotic Activation

The procedure followed was that of Appelmans and de Duve (1). The granules were submitted to gradient hypotonic sucrose solutions at 0° C. for a period of 15 min. before being incubated at 37° C. for 10 min., in presence of substrates containing enough sucrose to make the concentration of this sugar equal to 0.25 *M*. The results of a typical experiment are shown in Fig. 1.

Activation by Sodium Chloride

The granules were kept at 0° in 0.15 *M* sodium chloride and the free activities were measured at regular intervals. Free activities were determined concomitantly on part of the granules kept at 0° C. in 0.25 *M* sucrose. The

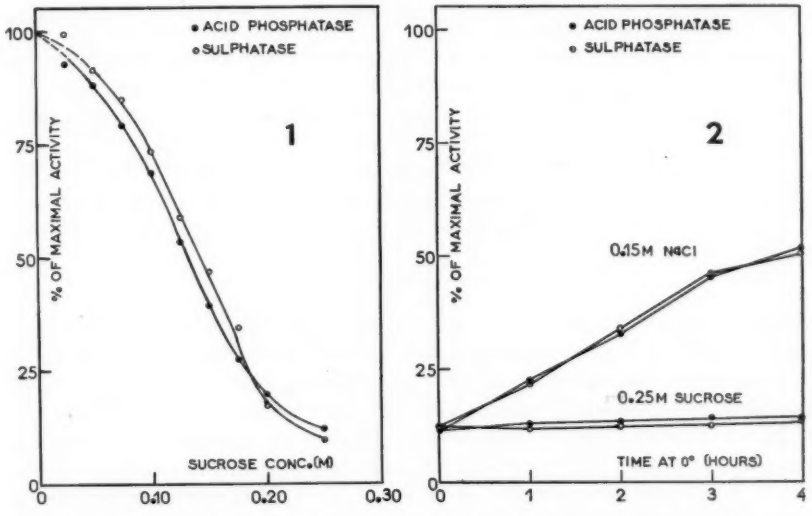


FIG. 1. Parallel release of acid phosphatase and 2-hydroxy-5-nitrophenylsulphate-specific sulphatase by gradient hypotonic sucrose solutions.

FIG. 2. Simultaneous liberation of acid phosphatase and 2-hydroxy-5-nitrophenylsulphate-specific sulphatase by 0.15 *M* sodium chloride.

concentrations of sodium chloride and sucrose in the substrates, determined according to the directions of Appelmans and de Duve (1), were such that the respective concentrations of the substances were 0.15 *M* and 0.25 *M* during the enzyme assays. As shown by Berthet *et al.* (3) and Appelmans and de Duve (1), the acid phosphatase-containing granules do not retain their integrity when kept at 0° in isotonic sodium chloride, whereas they remain stable at the same temperature in 0.25 *M* sucrose. Fig. 2 confirms these findings and indicates that bound sulphatase behaves similarly.

Mechanical Disruption

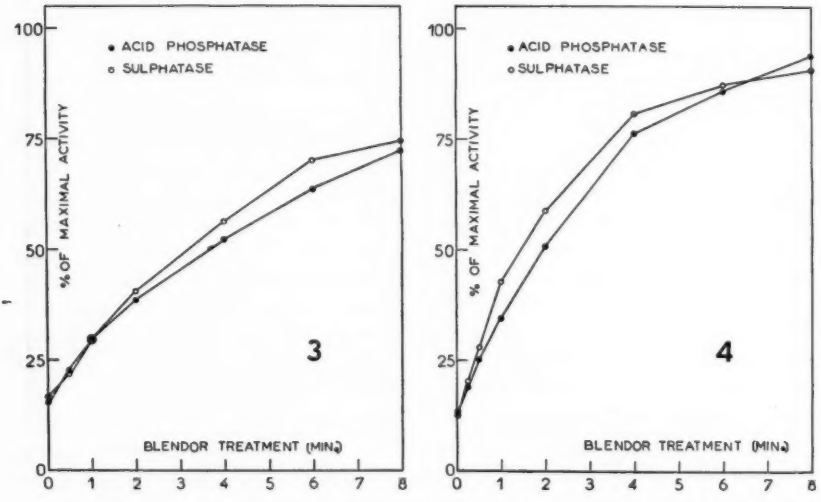
The free activities were made on a suspension of the granules in 0.25 *M* sucrose exposed to the action of a Waring blendor for increasing intervals of time. Fig. 3 shows the results of an experiment in which the Waring blendor was run at 40 v. while Fig. 4 expresses the results of an experiment in which the blendor was run at 90 v. instead of 110 v.

Activation by Freezing and Thawing

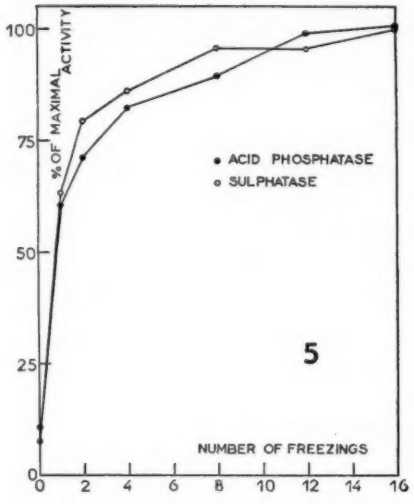
The granules were kept in 0.25 *M* sucrose, frozen in an ice–salt mixture, and thawed at 37° C. an increasing number of times. Fig. 5 illustrates the results of such an experiment.

Thermal Activation

The granules were suspended in 0.25 *M* sucrose containing 0.1 *M* acetate buffer at pH 5.5 and preincubated at 37° C. for periods up to three hours. Free enzymic activities were estimated at regular intervals by 10 min. incubation of these granules in the presence of substrate containing 0.25 *M* sucrose.



FIGS. 3 and 4. Mechanical disruption of the granules treated in a Waring blendor operated at 40 v. and 90 v. respectively.



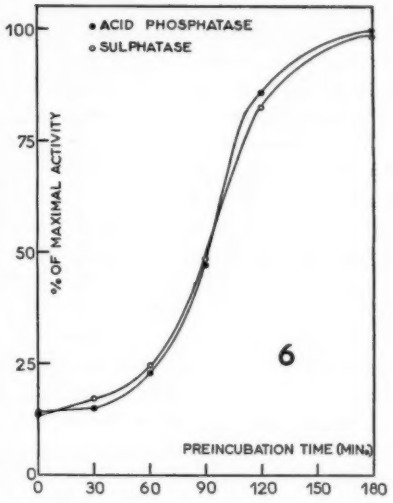


FIG. 5. Effect of freezing and thawing on the release of acid phosphatase and 2-hydroxy-5-nitrophenylsulphate-specific sulphatase.

FIG. 6. Influence of preincubation at 37° C. and pH 5.5 on the release of acid phosphatase and 2-hydroxy-5-nitrophenylsulphate-specific sulphatase.

However, since sulphatase is deactivated at 37° C. and pH 5.5, determinations of the total sulphatase activities were made at different intervals during the experiment. Portions of the granules were incubated for one, two, and three hours, after which total sulphatase activities of these preincubated suspensions were obtained by addition of substrate containing 0.15% triton x-100. The latter is a surface active agent which causes complete liberation of sulphatase from the granules to which it is bound.

Fig. 6 shows that complete activation of the granules is reached after three hours and that both enzymes are released simultaneously.

Discussion

As shown by the afore-mentioned results, the cytological properties of the 2-hydroxy-5-nitrophenylsulphate-specific sulphatase bear a striking resemblance to those of acid phosphatase. When in the bound state, this arylsulphatase exhibits only a part of its total activity, and the granules with which it is associated are irreversibly activated by methods which solubilize the enzyme: exposure to hypotonic sucrose solutions, treatment with salt solutions, mechanical disruption, freezing and thawing, and incubation at 37° C. in isotonic sucrose solutions. These are the same methods which de Duve and co-workers (1, 3, 4) have shown to activate the acid phosphatase-bearing granules of the rat-liver cell.

Roy (14) and Dodgson *et al.* (7) have claimed this arylsulphatase to be contained in the mitochondria fraction of rat liver. However the intracellular distribution they reported for this enzyme resembles much more that of acid phosphatase than that of cytochrome oxidase which may be considered a true mitochondrial enzyme.

Furthermore de Duve *et al.* (5) have pointed out that the intracellular localization of a given enzyme in the mitochondria must remain uncertain until the enzyme has been identified as belonging to either the "heavy" mitochondria (the cytochrome oxidase-bearing mitochondria), or the "light" mitochondria (the acid phosphatase-containing granules).

Intracellular distribution studies of this sulphatase as compared to that of acid phosphatase would have given little information regarding its real localization within the rat-liver cell on account of the non-proportionality between the quantity of 4-nitrocatechol liberated and the amount of tissue used.

However, the results reported here on the comparative binding experiments of the 2-hydroxy-5-nitrophenylsulphate-specific sulphatase and acid phosphatase indicate that both enzymes are associated with the same granules.

The initial free activities of both enzymes are very similar in each experiment although the sulphatase activity curve is somewhat shifted with respect to the other in some cases. This shifting is probably due to an error in the estimation of the total sulphatase activity arising from a slight denaturation of the enzyme on standing at 0° C. after blendor treatment.

A chemical link between the two enzymes would afford an obvious explanation for the observed analogy between their behaviors. However, Roy has shown by electrophoretic studies that 2-hydroxy-5-nitrophenylsulphate-specific sulphatase and acid phosphatase are distinct enzymes (15).

References

1. Appelmans, F. and de Duve, C. Biochem. J. (London), 59 : 426. 1955.
2. Appelmans, F., Wattiaux, R., and de Duve, C. Biochem. J. (London), 59 : 438. 1955.
3. Berthet, J., Berthet, L., Appelmans, F., and de Duve, C. Biochem. J. (London), 50 : 182. 1951.
4. Berthet, J. and de Duve, C. Biochem. J. (London), 50 : 174. 1951.
5. de Duve, C., Gianetto, R., Appelmans, F., and Wattiaux, R. Nature, 172 : 1143. 1953.
6. de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F. Biochem. J. In press. 1955.
7. Dodgson, K. S., Spencer, B., and Thomas, J. Biochem. J. (London), 56 : 177. 1954.
8. Dodgson, K. S., Spencer, B., and Thomas, J. Biochem. J. (London), 59 : 29. 1955.
9. Fiske, C. H. and Subbarow, Y. J. Biol. Chem. 66 : 375. 1925.
10. Gianetto, R. and de Duve, C. Biochem. J. (London), 59 : 433. 1955.
11. Gianetto, R. and Viala, R. Science, 101 : 801. 1955.
12. Hers, G., Berthet, J., Berthet, L., and de Duve, C. Bull. soc. chim. biol. 33 : 21. 1951.
13. Roy, A. B. Biochem. J. (London), 53 : 12. 1953.
14. Roy, A. B. Biochim. et Biophys. Acta, 14 : 149. 1954.
15. Roy, A. B. Biochem. J. (London), 57 : 465. 1954.

THE EFFECTS OF ADRENAL CORTICAL EXTRACTS UPON CARBOHYDRATE METABOLISM WHEN MEDIUM COMPOSITION IS ALTERED[1]

By D. W. Clarke

Abstract

The effects of adrenal cortical extract upon glucose uptake and upon glycogen synthesis in the isolated rat diaphragm were studied in three different media. The maximum inhibition by the extract upon the measured effects was seen in a medium which contained both sodium and potassium ions. In media which contained either of these alone as the major cation, there was no significant inhibition.

It is well established that various adrenal cortical steroids have an inhibiting action upon the glucose uptake and the glycogen synthesis of an isolated diaphragm incubated in an appropriate medium (2, 6, 7, 9). Some experiments of ours had previously shown that the ionic composition of the medium could also exert a strong influence upon glucose uptake and glycogen synthesis, and we therefore felt that it would be of interest to determine what effect these alterations in the composition of the medium had upon the effect of cortical extracts upon carbohydrate metabolism (3).

Experimental

Male white rats of 100–150 gm. were fasted for 20–24 hr. They were then decapitated and the diaphragms were removed. Tissues were soaked in a buffer only (no glucose, insulin, or cortical steroid) for 15 min. prior to being trimmed and weighed, according to the suggestion of Brown *et al.* (1). The compositions of the buffers used are shown below. The central portion was then excised and discarded, and two small portions from each side were removed, blotted, weighed, and put into hot potassium hydroxide for a determination of the initial glycogen. One of the remaining "halves" was placed in 2 ml. of incubation medium, which contained 200 mgm. % of glucose. The other "half" was placed in a similar medium, but in addition, insulin had been added to a final concentration of 1 unit/ml. In approximately half the experiments, adrenal cortical extract (ACE) was added to each of the above flasks. Three types of media were used, as indicated below. In one, the major cation was sodium; in another there was a sodium–potassium mixture; and in the third there was only potassium as the major cation.

After incubation at 37° for one hour, in stoppered 50 ml. Erlenmeyer flasks, with shaking, the tissues were removed and the glycogen in them was determined according to the procedure of Good, Kramer, and Somogyi (4). Aliquots were removed from the incubation medium for a determination of glucose, according to the procedure of Somogyi (8).

[1] *Manuscript received April 29, 1955.*
Contribution from the Department of Physiology, University of Toronto, Toronto, Ontario.

Materials

Buffers: "Sodium"—	NaCl	0.087 *M*
	$MgCl_2$	0.005 *M*
	pH	6.80
	Na_2HPO_4	0.040 *M*
"Mixed"—	KCl	0.087 *M*
	$MgCl_2$	0.005 *M*
	pH	6.80
	Na_2HPO_4	0.040 *M*
"Potassium"—	KCl	0.087 *M*
	$MgCl_2$	0.005 *M*
	pH	6.80
	K_2HPO_4	0.040 *M*

The pH of all buffers was adjusted to the value shown by the addition of hydrochloric acid.

Glucose: Added to buffers to give a final concentration of 200 mgm.%.

Insulin: Zinc insulin crystals prepared by Connaught Laboratories, Toronto, put in vials containing 40 units/ml. One-twentieth milliliter added to 2 ml. buffer to give a final concentration of 1 unit/ml.

Adrenal Cortical Extract (ACE): Vials of 50 units/ml. by Connaught Laboratories, Toronto. One-tenth of a milliliter of extract added to appropriate incubation flasks.

Results

A summary of the results concerning glycogen synthesis is shown in Table I. Glucose uptake values are summarized in Table II. The "insulin effect" is the difference between values obtained from insulin-treated diaphragms and those obtained from diaphragms which had not been treated with insulin. The values shown are the means of determinations on *N* animals, ± the standard error of the means. A negative value for glycogen synthesis means that the final glycogen content was less than the initial glycogen content.

Discussion

These results show that the inhibitory effect of ACE upon glycogen synthesis or glucose uptake depends to some extent upon the composition of the medium in which the experiment is performed. It is most interesting to note, however, that this inhibitory effect is only seen in a significant amount in the "mixed" medium in which both Na^+ and K^+ are present. If the medium contains only one of these cations, there is no significant effect of the ACE. It is not known which of the components of the ACE may be most responsible for this action, and further experiments are planned in which purified cortical extracts will be used.

TABLE I

	Glycogen synthesis, mgm./gm. tissue		Significance of difference
	With ACE	Without ACE	
Buffer—NaCl: Na_2HPO_4	*(N = 10)*		
Without insulin	0.21 ± .07	0.24 ± .11	NS
With insulin	0.64 ± .14	0.84 ± .14	NS
Insulin effect	0.42 ± .09	0.60 ± .12	NS
Buffer—KCl: Na_2HPO_4	*(N = 12)*	*(N = 11)*	
Without insulin	− 0.06 ± .08	0.15 ± .09	NS
With insulin	0.27 ± .12	0.84 ± .12	†
Insulin effect	0.33 ± .07	0.69 ± .11	*
Buffer—KCl: K_2HPO_4	*(N = 8)*		
Without insulin	− 0.32 ± .17	− 0.10 ± .15	NS
With insulin	− 0.21 ± .22	0.07 ± .14	NS
Insulin effect	0.11 ± .06	0.17 ± .04	NS

NS Not significant.
** Significant at 2% level or better.*
† Significant at 1% level or better.

TABLE II

	Glucose uptake, mgm./gm. tissue		Significance of difference
	With ACE	Without ACE	
Buffer—NaCl: Na_2HPO_4	*(N = 10)*		
Without insulin	2.70 ± .45	3.28 ± .56	NS
With insulin	4.62 ± .44	5.05 ± .62	NS
Insulin effect	1.91 ± .46	1.77 ± .22	NS
Buffer—KCl: Na_2HPO_4	*(N = 13)*		
Without insulin	2.19 ± .09	2.69 ± .31	NS
With insulin	2.64 ± .11	4.04 ± .41	†
Insulin effect	0.46 ± .12	1.35 ± .22	†
Buffer—KCl: K_2HPO_4	*(N = 8)*		
Without insulin	1.79 ± .27	1.51 ± .14	NS
With insulin	2.32 ± .21	2.39 ± .25	NS
Insulin effect	0.53 ± .09	0.88 ± .23	NS

NS Not significant.
† Significant at 1% level or better.

The inhibitory effect is seen only in those cases where insulin was added. This may be for two reasons:

1. The glycogen synthesis and glucose uptakes without added insulin are relatively low, and any differences are therefore small. Even if a real difference did exist, it may not have been statistically shown because of the relative magnitude of the standard error.
2. If ACE acts upon insulin to inhibit its action, then there will be no effect noted if no insulin is present.

Since, in most cases with no insulin treatment, the values obtained without ACE are higher than those obtained with ACE, there is the suggestion that the first explanation probably describes the main reason for the lack of significance.

It may also be seen from the tables that the glycogen synthesis and glucose uptake, both with and without insulin, decrease as the potassium concentration is increased. This confirms the work which was presented and discussed in a previous paper (3).

These results serve to emphasize the importance of the composition of the medium in experiments of this type. None of the media used in this work has a physiological composition, and in this respect it should be noted that the "sodium" medium corresponds to a medium which has been used quite extensively in recent years by various investigators. It seems probable that a medium with at least a small amount of potassium would be desirable. Verzar *et al.* (7, 9) obtained inhibitory effects with steroids in a medium which had a total sodium concentration of 0.15 *M* and a total potassium concentration of 0.004 *M*. In view of Hastings' results (5) though, it may be that the best potassium concentration is considerably higher than physiological.

Other anions or cations which were deliberately omitted from the media used in these experiments may similarly have a considerable influence upon the results of other experiments in which different factors are studied.

Acknowledgments

The author wishes to acknowledge the competent technical assistance of Mr. Haluk Senman and Mr. G. Urbach. The work was supported by a grant from the National Research Council of Canada.

References

1. Brown, D. H., Park, C. R., Daughaday, W. B., and Cornblath, M. J. Biol. Chem. 197 : 167. 1952.
2. Calkins, E. and Taylor, I. A symposium on the clinical and biochemical aspects of carbohydrate utilization in health and disease. *Edited by* V. Najjar. The Johns Hopkins Press, Baltimore, Md. 1952.
3. Clarke, D. W. Can. J. Biochem. Physiol. 33 : 687. 1955.
4. Good, C. A., Kramer, M., and Somogyi, M. J. Biol. Chem. 100 : 485. 1933.
5. Hastings, A. B., Teng, C.-T., Nesbett, F. B., and Sinex, F. M. J. Biol. Chem. 194 : 69. 1952.
6. Huisman, T. H. J. Acta Endocrinol. 13 : 836. 1949.
7. Leupin, E. and Verzar, F. Nature, 163 : 836. 1949.
8. Somogyi, M. J. Biol. Chem. 160 : 69. 1945.
9. Verzar, F. and Wenner, V. Biochem. J. 42 : 48. 1948.

BIOLOGICAL ASSAY OF THE TOXIN FROM SHELLFISH[1]

By N. R. Stephenson, H. I. Edwards, B. F. MacDonald, and L. I. Pugsley

Abstract

A conventional quantal response assay procedure was employed to determine the LD_{50} of shellfish extracts. The slope of the log dose – response line obtained with butter clam extracts was not significantly different from that found with scallop liver extracts. When kept in a dry, cold state, a lyophilized extract of scallop liver was stable throughout a test period of nearly three years. Such a preparation could be used as a reference standard for the bio-assay of shellfish toxin. Female mice were more susceptible to the paralytic poison than male mice. The LD_{50} per mouse was related directly to the average body weight of the test animals. The volume of the injection medium had no effect on either the magnitude of the LD_{50} or the slope of the probit regression line. A highly significant inverse relationship was demonstrated between the toxicity measured in mouse units, a procedure based on the mean death time of the test animals, and the LD_{50} per kgm. of mouse determined by an assay with an all-or-none response.

Introduction

Certain dinoflagellate plankton of the *Gonyaulax* species have been shown to be a primary source of the paralytic toxin found in shellfish (4, 7). Extracts containing this toxic principle have been prepared and concentrated to a relatively high degree of purity (1, 9, 10). Until more information is available on the chemical structure and properties of the toxin, the determination of the toxin from shellfish, which is important from a public health viewpoint, must be performed by means of a suitable biological assay.

The method currently employed for the routine assay of shellfish extracts is a modification of a procedure originally described by Sommer and Meyer (8). An extract is prepared from the species of shellfish under investigation and a 1 ml. aliquot is injected intraperitoneally into each of three white mice weighing 18 to 22 gm. The time from the injection until death is measured to the nearest five seconds, and the mean death time of the test animals is referred to a standard curve from which the toxicity of the unknown extract is determined and expressed in "mouse units" per 100 gm. of shellfish. A mouse unit is the amount of toxin in 1 ml. of an extract which will kill a 20 gm. mouse in from 15 to 20 min. with symptoms of paralysis or respiratory failure (3, 6).

The mouse unit is related to the minimal lethal dose of the paralytic toxin and as such assumes that all of the mice have the same tolerance to the poison and neglects the variation in susceptibility which is known to exist in a normal population of test animals (11). The most acceptable method for determining the amount of toxin in a shellfish extract is to compare the activity of the unknown with that of a standard preparation. Unfortunately at present a reference standard for shellfish toxin has not been adopted and therefore it is not possible to determine the toxicity in this manner.

[1] *Manuscript received May 20, 1955.*
Contribution from the Department of National Health and Welfare, Food and Drug Laboratories, Ottawa and Vancouver, Canada.

This paper is concerned with the measurement of the median lethal dose (LD_{50}) of a shellfish extract by means of an assay with an all-or-none response. A method was investigated for preparing a stable reference standard for the paralytic toxin, and a study was made of various factors affecting its bio-assay. Finally the toxicity of a shellfish extract, measured in mouse units and based on the mean death time of the test animals, was compared with that estimated by the LD_{50} determined by means of a quantal response type of assay.

Methods

Preparation of the Shellfish Extracts

The shellfish extracts used in this investigation were prepared according to the procedure outlined by Medcof *et al.* (3). The Food and Drug Laboratories in Vancouver, B.C., employed the butter clam (*Saxidomus giganteus*) as the source of the shellfish toxin. The acid–aqueous extract of butter clams was prepared, clarified by centrifugation, sealed in small ampoules, autoclaved at 15 lb. pressure for 15 min., and stored in the dark at room temperature. The sterile ampoules were used as required in the tests performed by the Vancouver laboratory.

An acid–aqueous extract of scallop (*Pecten grandis*) liver was prepared at the Atlantic Biological Station, St. Andrews, N.B., and shipped to the Food and Drug Laboratories, Ottawa, Ontario, for assay. This scallop liver extract was lyophilized, reduced to a fine powder, and stored in the dry state in the refrigerator. The powder was found to contain 6.5% total nitrogen (micro-Kjeldahl) and 9.4% moisture (dried to constant weight at 100°). Portions of the powder were weighed and dissolved in water for the assays conducted in the Ottawa laboratory.

Assay Procedure

Albino mice of either sex, weighing 20–30 gm., were used as the test animals for the determination of the median lethal dose (LD_{50}) of the toxic material in the shellfish preparations. A preliminary test was conducted with each extract to ascertain the dosage level which would kill approximately one-half of the mice in the group between 3 and 20 min. after the intraperitoneal injection. This was accomplished by starting with a definitely lethal dose and gradually decreasing the amount of the extract in the injection medium until the desired range of dosage levels was reached. A series of three to four doses were selected to produce percentage kills ranging from 10% to 90%. Dose levels were avoided which would cause either no deaths or 100% mortality. The volume of the solution injected intraperitoneally was varied from 0.25 ml. to 1.0 ml., but for routine use either 0.5 ml. of the scallop liver extract or 1.0 ml. of the butter clam extract was administered intraperitoneally to the mice.

In the morning of the assay day, a group of either male or female mice were weighed to the nearest gram and placed into groups according to their body weight. The weight range of the mice to be used in the test was kept as

narrow as possible and the average body weight of the mice in each dosage group was equalized by the appropriate distribution of the mice from each weight group into the required number of dosage groups. The various dose levels were assigned at random to the dosage groups, each of which contained at least 10 mice.

After the extract was administered by intraperitoneal injection, the mice were placed in liter beakers, either individually or in pairs, and the time of injection noted. The percentage mortality, observed over a period ranging from 30 min. to three hours after injection, was recorded for each group. As noted previously by Sommer and Meyer (8) most of the deaths occurred less than 30 min. after the administration of the toxic material.

The percentage kill at each dosage level was converted to probits and the LD_{50} was estimated by the method described by Finney (2).

In addition to the quantal response assay, in some of the tests with butter clam extracts, the toxicity was determined by the modified Sommer and Meyer procedure (3, 8), which is based on the average death time of the mice, and expressed in mouse units per 100 gm. of shellfish tissue.

Results and Discussion

The Log Dose – Response Relationship

Fig. 1 shows that a straight line was obtained when the probit of the percentage kill was plotted against the logarithm of the dose of the scallop liver extract. In this typical assay the slope of the regression line, calculated

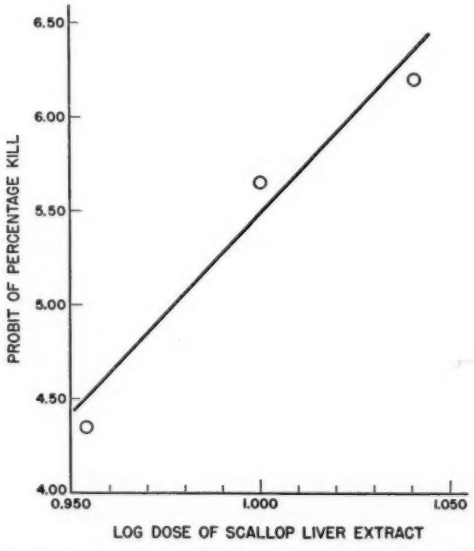


FIG. 1. A typical log dose – response line for the scallop liver extract; 35 female mice in each dosage group.

by the method of least squares, was 22.0 with a standard deviation of ± 4.0. The Chi-square test for heterogeneity was not significant when applied to these data indicating no systematic deviation from linear regression. In a series of 19 assays of scallop liver extracts, the mean slope of the log dose – response relationship and its standard error was found to be 20.19 ± 1.43. It is obvious from the low value of the standard error that the slopes of the individual probit regression lines were quite uniform.

Influence of the Sex of the Test Animal on the LD_{50} of the Shellfish Toxin

Table I shows the LD_{50} values and the slopes of the probit regression lines for both male and female albino mice. The LD_{50} of the toxin from both the butter clams and the scallop livers was lower when the female mice were used as the test animals than it was when males were employed. This difference was significant ($P = 0.05$) according to the "t" test and indicated that female mice were more susceptible to the toxic material than males. Although the average body weight of the males was not actually significantly higher than that of the females in each group, the sex difference observed may have been influenced to some extent by the body weight. The slopes of the probit regression lines were not affected significantly by the sex of the test animal according to the Chi-square test.

TABLE I

THE EFFECT OF SEX ON THE LD_{50} AND THE SLOPE OF THE LOG DOSE – RESPONSE LINE

Source of toxin	Sex	No. of mice	LD_{50} per mouse	Fiducial limits ($P = 0.05$)	Slope "b"	Average body wt., gm.
Butter clam	Male	229	0.141 cc.	0.138–0.144	18.44	20.8
	Female	224	0.127 cc.	0.125–0.130	22.50	20.0
Scallop liver	Male	369	1.182 mgm.	1.162–1.202	18.84	25.8
	Female	849	1.034 mgm.	1.022–1.046	19.21	23.6

When the data for the males and the females were pooled by Perry's method (5), the slope of the log dose – effect line for the butter clam extract was not significantly different from that calculated for the scallop liver extract. This observation suggested that the physiological action of the paralytic toxin found in butter clams is identical to that obtained from scallop liver. If the toxins from other species of shellfish show a similar tendency, the toxicity of extracts from different types of shellfish may be determined in terms of a single reference standard.

The Effect of the Body Weight on the LD_{50} and the Toxicity Estimated in Mouse Units

The median lethal dose of the shellfish extract was plotted against the average body weight of the mice used in the assay. The scatter diagrams

obtained for the butter clam extract and the scallop liver extract are shown in Figs. 2 and 3 respectively, and demonstrate that a significant relationship exists between the LD_{50} of the extract and the average body weight of the test animals.

In order to make an allowance for the effect of the body weight on the LD_{50}, the toxicity of the extracts was expressed as the LD_{50} per kgm. of mouse. However, when 30 gm. mice were used to estimate the LD_{50} per kgm., the value obtained was usually somewhat smaller than that found when 20 gm. mice were employed for this purpose. Although the correlation coefficient relating the LD_{50} per kgm. and the average body weight was not significant, it had a negative value, indicating that the LD_{50} per kgm. tended to decrease as the average body weight increased. Thus it is apparent that the estimate

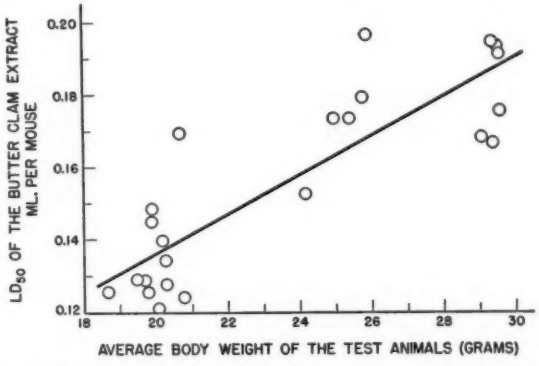


FIG. 2. The relationship between the LD_{50} of the butter clam extract and the average body weight of the mice; $r = 0.856$ ($P < 0.001$).

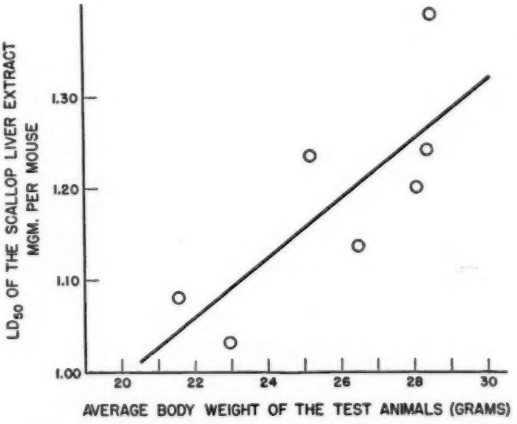


FIG. 3. The relationship between the LD_{50} of the scallop liver extract and the average body weight of the mice; $r = 0.789$ ($P < 0.05$).

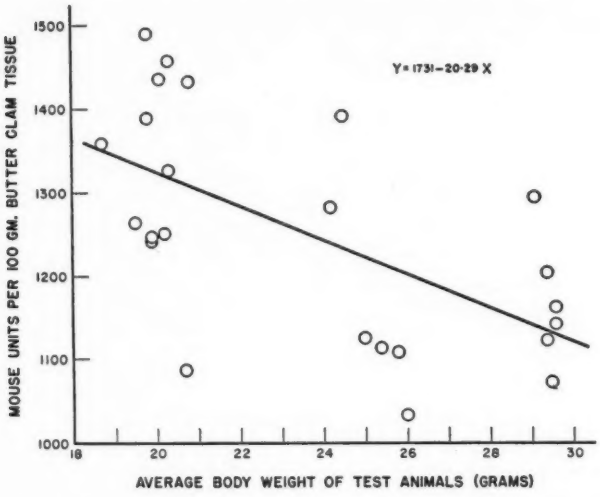


FIG. 4. The relationship between the toxicity of the butter clam extract estimated in mouse units per 100 gm. of tissue and the average body weight of the mice.

of the toxicity, even when measured as the LD_{50} per kgm., is dependent not only on the amount of toxin in the shellfish extract but also on the average body weight of the test animals.

It was frequently noted that replicate estimates of toxicity in terms of mouse units lacked uniformity when mice of widely varying weights were used, although the weight correction factors proposed by Sommer and Meyer (8) had been employed. This observation is represented graphically by the scatter diagram in Fig. 4 where the mean toxicity, derived from individual estimates which had been corrected for body weight, was plotted against the mean weight of the animals in the assay. The significant correlation coefficient ($r = -0.601$) indicates that an inverse relationship between body weight and toxicity in mouse units still persists, in spite of the application of the correction factors. The degree of correction thus provided is insufficient and some modification is therefore necessary. From these data it has been determined that correction of the original weight conversion factors by the addition of approximately 1.6% of their value for each gram of mouse body weight, in excess of 20 gm., will result in improved estimates of toxicity.

The Effect of the Volume of the Injection Medium on the LD_{50}

An aliquot of the extract from butter clams was divided into two portions in such a way that the doses could be administered in a volume of either 0.25 ml. or 1.0 ml. The data from four assays, yielding a total of 172 mice at each dilution, were combined according to the method described by Perry (5) to give the results shown in Table II.

TABLE II

THE EFFECT OF THE VOLUME OF THE SOLUTION INJECTED ON THE LD_{50} AND THE SLOPE OF THE LOG DOSE – RESPONSE LINE

Volume of solution injected, ml.	LD_{50} per mouse, ml.	Fiducial limits ($P = .05$), ml.	Slope "b"
1.0	0.155	0.151–0.159	18.52
0.25	0.156	0.151–0.161	16.62

According to these data the volume of the injection within the limits of 0.25 and 1.0 cc. had no effect on the LD_{50} of the paralytic toxin derived from butter clams. In addition, there was no significant difference between the slopes of the probit regression lines.

Stability of the Paralytic Shellfish Poison

The stability of both the autoclaved butter clam extract and the lyophilized scallop liver extract was evaluated by tabulation of the values obtained for the LD_{50} per kgm. of mouse in chronological order. Fig. 5 presents these data plotted as a scatter diagram and illustrates the effect of time on the LD_{50} of each of the shellfish preparations. The LD_{50} of the autoclaved extract increased with time and the correlation coefficient was highly significant ($r = 0.79$). On the other hand the LD_{50} of the lyophilized extract of scallop livers, although erratic over the 133 week period of the test, did not exhibit such an increase with time. The correlation coefficient in this case was not significant ($r = -0.12$).

Apparently the toxic principle from butter clams disappeared slowly from the autoclaved acid–aqueous extract. On the other hand, when kept as a

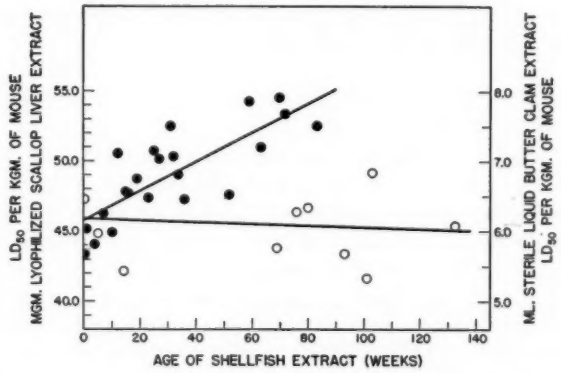


FIG. 5. The relationship between the LD_{50} per kgm. of mouse and the age of the shellfish extract.
Black circles—butter clam extract. Open circles—scallop liver extract.

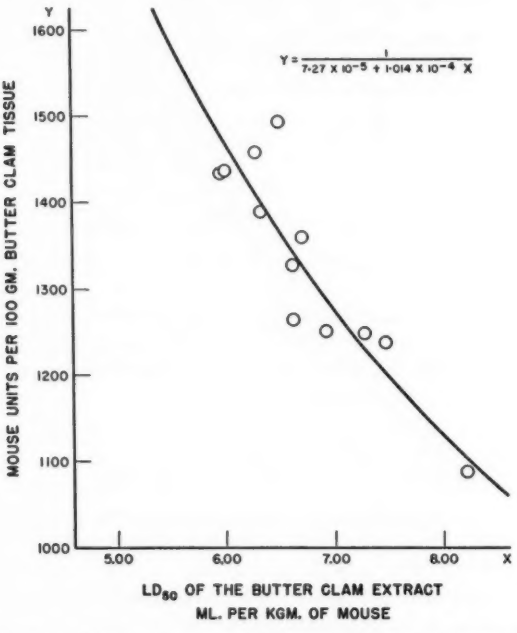


FIG. 6. The relationship between mouse units per 100 gm. of butter clam tissue and the LD_{50} per kgm. of mouse; $r = -0.892$ ($P < 0.001$).

dry powder in the cold, the toxin in scallop livers remained at the same level of activity during the entire test period of nearly three years. Lyophilization of the aqueous extract of a shellfish toxin followed by storage in the cold provided a satisfactory procedure for preserving the paralytic poison in a stable state. Therefore a procedure such as this could be used for preparing an acceptable reference standard for shellfish toxin.

The Relationship Between Mouse Units per 100 gm. of Shellfish Tissue and the LD_{50} per kgm. of Mouse

When the toxicity of butter clam extracts was determined by both the quantal response method and the modified Sommer and Meyer procedure (3, 8), the highly significant inverse relationship shown in Fig. 6 was found to exist between the two values obtained. From this regression line it was possible to predict the number of mouse units per 100 gm. of butter clam tissue from the LD_{50} per kgm. of mouse and vice versa. Although both procedures appear to measure toxicity equally well, the quantal response assay has the distinct advantage that fiducial limits can be calculated for the LD_{50} per kgm. of mouse.

In addition, the quantal response assay employed in this investigation can be easily adapted to a procedure in which the toxicity of a shellfish extract is

measured in terms of a reference standard. Since the toxic material in both the butter clams and the scallop livers produced probit regression lines with similar slopes it should be possible to set up a lyophilized extract from one or more species of shellfish as the reference standard. Factors such as the sex and the body weight of the test animals, which have been shown to have a significant effect on both the LD_{50} and the mouse unit, would have no influence on the toxicity determined in this manner.

Acknowledgment

The authors wish to thank Dr. Medcoff and his co-workers of the Atlantic Biological Station, St. Andrews, N.B., for preparing the scallop liver extract used in this study and are indebted to Mr. A. J. Whitmore, Chief Supervisor of Fisheries, Vancouver, B.C., and his inspection staff for providing the shellfish samples from which the butter clam extract was prepared. In addition, they wish to acknowledge the valuable technical assistance of Mr. P. J. Kavanagh, Miss P. Palamaryk, and Mr. J. C. Taylor.

References

1. Bendien, W. M. and Sommer, H. Proc. Soc. Exptl. Biol. Med. 48 : 715. 1941.
2. Finney, D. J. Probit analysis. Cambridge University Press, London. 1947. pp. 48-63' 199-208.
3. Medcof, J. C., Leim, A. H., Needler, A. B., Needler, A. W. H., Gibbard, J., and Naubert, J. Fisheries Research Board Can. Bull. No. 75. 1947.
4. Needler, A. B. J. Fisheries Research Board Can. 7 : 490. 1949.
5. Perry, W. L. M. The design of toxicity tests. Med. Research Council (Brit.) Spec. Rept. Ser. No. 270. 1950.
6. Prinzmetal, M., Sommer, H., and Leake, C. D. J. Pharmacol. Exptl. Therap. 46 : 63. 1932.
7. Riegel, B., Stanger, D. W., Wikholm, D. M., Mold, J. D., and Sommer, H. J. Biol. Chem. 177 : 7. 1949.
8. Sommer, H. and Meyer, K. F. Arch. Pathol. 24 : 537. 1937.
9. Sommer, H., Monnier, R. P., Riegel, B., Stanger, D. W., Mold, J. D., Wikholm, D. M., and Kiralis, E. S. J. Am. Chem. Soc. 70 : 1015. 1948.
10. Sommer, H., Riegel, B., Stanger, D. W., Mold, J. D., Wikholm, D. M., and McCaughey, M. D. J. Am. Chem. Soc. 70 : 1019. 1948.
11. Trevan, J. W. Proc. Roy. Soc. (London), B, 101 : 483. 1927.

EFFECT OF STARVATION ON CHOLESTEROL BIOSYNTHESIS *IN VITRO*[1]

By B. B. Migicovsky and J. D. Wood

Abstract

The components of centrifugal fractionation of liver homogenates from normal and starved rats were examined for cholesterol synthesis and inhibition thereof. Normal liver particulate matter fractions obtained between 700 to 9000 × *g* and 9000 to 140,000 × *g* were active with respect to cholesterol synthesis in the presence of clear supernate obtained by centrifugation at 140,000 × *g*. The particles alone did not synthesize cholesterol. Particles from liver homogenate of starved rats, recombined with clear supernate from starved rats, lacked synthetic activity but clear supernate from liver homogenate of starved rats showed some activity in the presence of normal particles. Degradation of cholesterol occurred with liver particles (700–9000 × *g*) from both normal and starved rats: the latter preparation also inhibited cholesterol synthesis. Preliminary incubation of clear supernate from normal rats with particles from starved rats, followed by recentrifugation at 140,000 × *g*, produced a supernate with synthetic activity equal to that obtained with untreated supernate. Preliminary incubation with normal particles gave a supernate with less synthetic activity. This indicated another difference between particulate matter from normal and starved rats.

Introduction

Numerous investigators have observed the relationship between the physiological state of an animal and altered cholesterol metabolism (3, 4). *In vitro* studies (5, 6) have demonstrated a decrease in lipogenesis after short fasting periods. Whitney and Roberts (8) reported that a high fat diet prior to fasting stimulated cholesterol synthesis. A previous report from this laboratory (7) indicated that the liver homogenate from starved rats, prepared according to Bucher (1), had an inhibitory effect on cholesterol synthesis by a normal rat liver preparation.

This paper reports further investigations on the differences between preparations of livers from normal and starved rats.

Methods and Materials

The technique of measuring acetate incorporation was the same as reported previously (7). The liver homogenates were prepared according to Bucher (1) except that the liver was perfused with ice-cold 0.25 *M* sucrose prior to excision. The homogenization of the liver also differed in that it was not prolonged for more than one-half minute.

Incubations were carried out in 20 ml. beakers in a Dubnoff water bath under oxygen at 37° C. Duration of incubation was three hours except where otherwise stated. The incubation mixture consisted of 1.3 mgm. ATP, 3.8 mgm. DPN, 1.0 μmole acetate-1-C^{14} solution plus a liver preparation and pH 7.2 buffer prepared according to Bucher (1) to make a final volume of 2.4 ml.

[1] *Manuscript received May 16, 1955.*
Contribution No. 283 from the Chemistry Division, Science Service, Canada Department of Agriculture, Ottawa, Canada.

After incubation and prior to saponification, 5 mgm. of cholesterol in 5 ml. of alcohol was added to each beaker as carrier. The results are expressed as micromoles of acetate incorporated into the total cholesterol. This value is equivalent to $\frac{\text{counts per minute/mgm. digitonide}}{\text{counts per minute/}\mu\text{mole acetate}} \times 20$. The factor of 20 is the cholesterol digitonide equivalent of 5 mgm. of cholesterol.

Fractionation of the homogenate was carried out at 0° C. in a Spinco Model L ultracentrifuge. The cell-free homogenate system was prepared by centrifugation of the homogenized liver for 10 min. at 600 × *g*. The supernate, hereinafter referred to as complete homogenate, was separated into three fractions: (*a*) particles which sedimented after 15 min. at 600 to 9000 × *g*; (*b*) particles which sedimented after 30 min. at 9000 to 140,000 × *g*; (*c*) a clear supernate obtained from centrifugation at 140,000 × *g* for 30 min. Hereinafter the first sediment is referred to as residue 1, the second sediment as residue 2, and the supernate as clear supernate. We have observed that great care must be taken in reconstituting these fractions or activity will be lost. Excessive stirring, grinding, or homogenizing destroys the activity.

The rats used in these experiments weighed 120 to 175 gm. They were normally fed or starved for 48 hr. before their livers were excised. In the subsequent discussion, the symbols N and S are used to denote homogenate fractions prepared from livers of normal (N) or starved (S) rats.

Results

The object of the first series of experiments was to fractionate the system with respect to its ability to synthesize cholesterol. The results are shown in Table I. They indicate that both the residue 1 and residue 2 fractions were active when combined with clear supernate; either fraction by itself was inactive.

The absence of activity in preparations made from livers of starving rats was reported by Migicovsky (7) and is confirmed by the data presented in Table II. The results show that the S particulate matter, when recombined

TABLE I

INCORPORATION OF ACETATE INTO CHOLESTEROL BY VARIOUS FRACTIONS OF A NORMAL RAT LIVER HOMOGENATE

Homogenate fraction	μM. acetate incorporated × 10^3	
	Expt. 106	Expt. 105
Complete homogenate	49.2	37.2
Residue 1 plus clear supernate	21.7	18.4
Residue 2 plus clear supernate	16.2	15.0
Clear supernate	0.1	0

TABLE II

COMPARISON OF HOMOGENATE FRACTIONS FROM STARVED (S) AND NORMAL (N) RAT LIVERS

Homogenate fraction	μM. acetate incorporated $\times 10^3$		
	Expt. 109	Expt. 119	Expt. 122
N residue 1 plus N clear supernate	1.27	6.32	5.04
N residue 1 plus S clear supernate	0.22	1.74	1.0
S residue 1 plus N clear supernate	0	0	0
S residue 1 plus S clear supernate	0	0	0

with N or S clear supernate, did not support cholesterol synthesis. The S clear supernate, however, did support synthesis to a minor degree when it was recombined with N particulate matter.

The difference between N clear supernate and S clear supernate is further illustrated in Fig. 1. The curve indicates that replacement of N clear supernate by S clear supernate reduced the activity. The difference between the two supernates is quantitative with respect to some factor or factors.

Synthetic activity occurred only when both supernate and particles were present. This led to an investigation of the effect of the relative proportion of each. Results of this experiment are shown in Table III.

Increasing the ratio of particles to supernate raised the velocity of the synthetic reaction. After a short period of time, the presence of excess particles reduced the activity. A study of the time response indicates that the synthesized cholesterol was degraded by this preparation. It appears, then, that the homogenate system employed in the study of the synthesis of

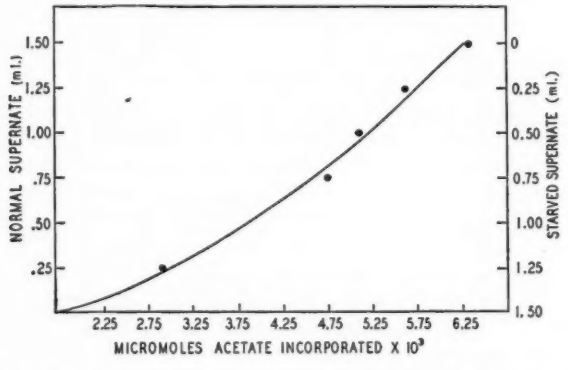


FIG. 1. The effect of replacing the clear supernate in a "normal" rat liver homogenate by clear supernate from a "starved" liver homogenate.

TABLE III

THE EFFECT OF INCREASING THE AMOUNT OF RESIDUE 1 ON ACETATE INCORPORATION INTO CHOLESTEROL

Equivalent amount* of residue 1 added to 1.5 ml. complete homogenate	Duration of incubation in minutes			
	40	60	90	120
	μM. acetate incorporated $\times 10^3$			
0	3.6	9.7	11.0	6.4
1	7.4	10.4	14.0	15.2
2	8.0	17.2	18.6	15.8
3	8.1	12.6	12.9	11.4

** An equivalent amount of residue is that quantity obtained from 1.5 ml. of complete homogenate.*

cholesterol is also capable of degrading cholesterol. The possibility exists also that the decreased activity caused by an excess of particulate matter may be due to an increased degradative activity.

The degradation of cholesterol is further illustrated in Table IV. In this experiment, the incubation of C^{14}-acetate with N homogenate was allowed to proceed for one and one-half hours, at which time the additions shown in the table were made. After two hours' further incubation, the radioactivity of the cholesterol from the different treatments was compared. The results show that cholesterol disappeared in all cases, especially after addition of DPN plus S complete homogenate.

TABLE IV

DISAPPEARANCE OF PREFORMED RADIOACTIVE CHOLESTEROL AFTER FURTHER INCUBATION FOR TWO HOURS

Addition to incubation mixture after 1.5 hr. incubation*	% preformed cholesterol lost after 2 hr. additional incubation	
	Expt. 81	Expt. 83
0.6 ml. buffer	27.4	23.8
0.5 ml. S complete homogenate	22.9	20.9
2.5 mgm. DPN	24.6	28.1
0.5 ml. S complete homogenate plus 2.5 mgm. DPN	35.2	39.0

** S = homogenate prepared from liver of a starved rat.*

In another experiment, radioactive cholesterol was suspended in water and added to the preparations noted in Table V. A considerable amount of activity was lost over a period of two hours, which demonstrated a degradative action on the part of the homogenate preparations. This degradative activity appeared to be the same with both N and S complete homogenates. The real difference between N and S preparations seems to lie in their inhibitory capacity, as was reported by Migicovsky (7).

TABLE V

DEGRADATION OF C^{14}-CHOLESTEROL BY VARIOUS LIVER HOMOGENATE PREPARATIONS

Homogenate prepared from	% C^{14}-cholesterol lost
Normal rat liver	23
Starved rat liver	17
Starved rat liver plus 2.5 mgm. DPN	26

The second series of experiments deal with the inhibitory phase of the problem. The inhibitory properties of S preparations are illustrated in Fig. 2. The curves show that addition of 0.5 ml. of S complete homogenate to 1.5 ml. of N complete homogenate decreased considerably the rate of acetate incorporation into cholesterol. The fact that the inhibitory activity was not present in the supernate is illustrated by the data shown in Table VI.

TABLE VI

NON-INHIBITORY ACTIVITY OF THE SUPERNATE FRACTION OF LIVER HOMOGENATE FROM STARVED RAT

Addition to 1.5 ml. complete homogenate from liver of normal rat*	μM. acetate incorporated $\times 10^3$	
	Expt. 108	Expt. 101
None	10.6	6.9
0.5 ml. N clear supernate	11.0	5.9
0.5 ml. S clear supernate	12.0	6.2
0.5 ml. S complete homogenate	—	2.1
0.33 equivalent† S residue 1	1.9	—

* *N = from liver of normal rat.*
S = from liver of starved rat.
† *An equivalent amount of residue is that quantity obtained from 1.5 ml. of complete homogenate.*

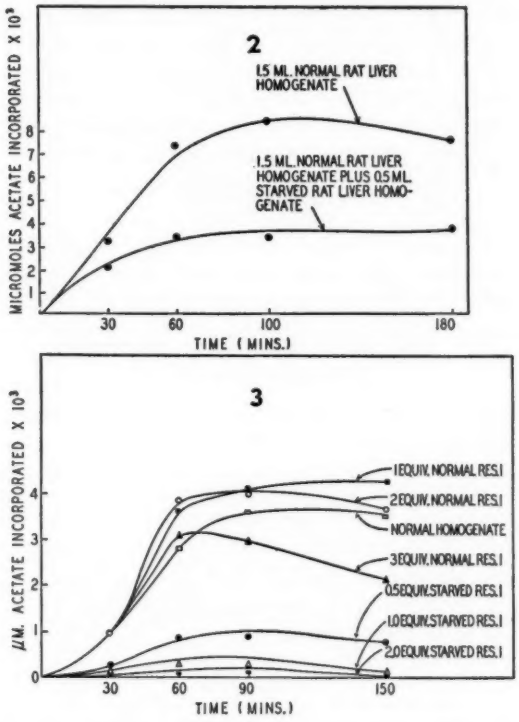


FIG. 2. Rate of $CH_3C^{14}OOH$ incorporation into cholesterol by "normal" rat liver homogenate and inhibition by "starved" rat liver homogenate.

FIG. 3. Effect of adding "normal" and "starved" residue 1 to "normal" rat liver homogenate.

The effect of additions of N and S residue 1 on the rate of acetate incorporation into cholesterol is shown in Fig. 3. The high degree of inhibition which occurred with S particulate matter was probably not a result of an increased rate of degradation of cholesterol. Three equivalents of N residue 1 caused a small apparent inhibition after two hours' incubation. Examination of the time response indicates that this apparent inhibition was due to degradation of the synthesized cholesterol.

The effect of acetate concentration on the inhibitory activity is shown in Fig. 4. In this experiment, the incubation was allowed to proceed for 60 min. It is apparent that increasing the acetate concentration increased the percentage inhibition.

As a result of these observations, it appeared possible that the S particulate matter was inactivating a factor present in the supernate. An experiment to test this was carried out and the results are shown in Table VII. The various supernates, N and S, were allowed to stand at room temperature for 45 min.

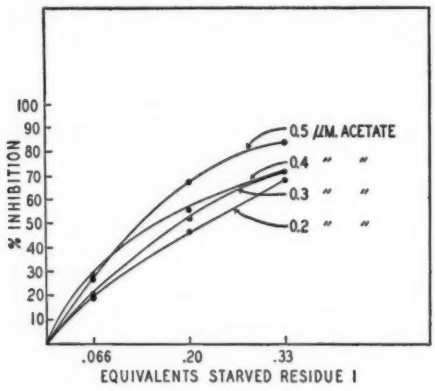


FIG. 4. Per cent inhibition of acetate incorporation into cholesterol by "starved" residue 1 at various concentrations of acetate.

with either S or N residue 1 in the absence of added acetate. After this period, the supernate was recovered by centrifugation and recombined with fresh N or S residue 1 and incubated with C^{14}-acetate for 90 min. to measure the degree of acetate incorporation into cholesterol. Results indicate that the first incubation of either N or S supernate with S particles in the absence of substrate did not affect the synthetic activity. Incubation of the supernates

TABLE VII

EFFECT OF PREINCUBATION OF CLEAR SUPERNATE WITH RESIDUE 1 OF LIVER HOMOGENATES PREPARED FROM NORMAL (N) AND STARVED (S) RATS

Preincubation* mixture	Recovered supernate† incubated with	μM. acetate incorporated × 10^3
N clear supernate	N residue 1	9.72
N clear supernate	S residue 1	0
N clear supernate plus N residue 1	N residue 1	5.46
N clear supernate plus S residue 1	N residue 1	8.44
S clear supernate plus N residue 1	N residue 1	1.60
S clear supernate plus N residue 1	S residue 1	0
S clear supernate plus S residue 1	N residue 1	2.65
——	Unincubated supernate plus N residue 1	9.41

* *Mixture was allowed to stand at room temperature for 45 min. without added acetate, ATP, or DPN.*

† *Incubation with radioactive acetate in usual manner for 90 min.*

with N particulate matter at room temperature caused a decrease in the synthetic activity of the supernates. These results negate the possibility mentioned above, but on the other hand, they illustrate another difference in behavior between N and S particulate matter. It seems that the N particulate matter did destroy, to some extent, a factor or factors in the supernate that were necessary for cholesterol synthesis.

Discussion

Fractionation of the liver homogenate with respect to cholesterol synthetic activity indicated that both clear supernate and particulate matter were necessary. Either fraction by itself was inactive. Migicovsky (7) reported that the supernate fraction which was obtained by centrifugation at 21,000 × *g* in a Servall centrifuge still retained some activity. This residual activity was likely due to incomplete removal of particulate matter.

Bucher *et al.* (2) reported that a sediment collected between 9000 and 100,000 × *g*, to which they referred as the microsome fraction, was active when combined with a clear supernate, whereas the sediment collected between 700 and 9000 × *g*, referred to as the mitochondrial fraction, was inactive. This observation is at variance with the results reported in this paper.

Comparison of S and N preparations led to the conclusions that S homogenates could not synthesize cholesterol but both N and S preparations were capable of degrading cholesterol. The data also demonstrated that the inability of the S preparation to synthesize cholesterol was due in part to some factor or factors missing in the supernate fraction.

Experiments on the inhibitory properties of S homogenates demonstrated that the S supernate did not inhibit. The inhibitory activity resided only in the particulate matter. Migicovsky (7) reported some inhibitory activity in the supernate obtained upon centrifugation at 21,000 × *g*. This activity was undoubtedly due to incomplete removal of particulate matter.

Summary and Conclusions

Empirical partition of the particulate matter by centrifugation of liver homogenates did not lead to a partitition of cholesterol synthetic activity.

The homogenate, whether prepared from a starved or normal rat, was capable of degrading cholesterol.

The homogenate from a starved rat differed from the preparations made from a normal rat in that (*a*) the S supernate was able to support cholesterol synthesis to a lesser degree than N supernate when it was recombined with N particulate matter: therefore, it was lacking in some factor or factors; (*b*) the S particulate matter was unable to support cholesterol synthesis and contained all the inhibitory activity of S homogenate; (*c*) incubation of N particulate matter with supernate effected a change in the supernate in that its synthetic capacity was diminished. No such change was effected by particulate matter obtained from the liver homogenate of a starved rat.

Acknowledgment

The valuable assistance of A. G. Ward and L. J. Carter is gratefully acknowledged.

References

1. Bucher, N. L. R. J. Am. Chem. Soc. 75 : 498. 1953.
2. Bucher, N. L. R., Grover, J. W., and Kingston, R. Federation Proc. 13 : 19. 1954.
3. Byers, O. S., Friedman, M., and Rosenman, R. H. Am. J. Physiol. 178 : 327. 1954.
4. Hutchens, T. T., Van Bruggen, J. T., Cockburn, R. M., and West, E. S. J. Biol. Chem. 208 : 115. 1954.
5. Lyons, I., Masri, M. S., and Chaikoff, I. L. J. Biol. Chem. 196 : 25. 1952.
6. Medes, G., Thomas, A., and Weinhouse, S. J. Biol. Chem. 197 : 181. 1952.
7. Migicovsky, B. B. Can. J. Biochem. Physiol. 33 : 135. 1955.
8. Whitney, J. E. and Roberts, S. Federation Proc. 13 : 320. 1954.

CARDIAC AUTOMATISM AND CHOLINE ACETYLATION IN RATS ON THIAMINE- AND PANTOTHENIC-ACID-DEFICIENT DIETS[1]

By A. B. L. Beznák and G. W. H. M. van Alphen

Abstract

The choline acetylating activity of extracts of the acetone powders of hearts of rats fed on diets deficient in thiamine, in pantothenate, and in thiamine plus pantothenate was found to be the same as it was in rats on a complete synthetic diet, although severe changes in the electrocardiogram (e.c.g.) were present in rats on the two thiamine-deficient diets. These e.c.g. changes were the same as the ones described in rats on diets deficient in other factors besides thiamine. The rats on the pantothenic-acid-deficient diet grew as normals did and no e.c.g. abnormalities were seen in them. The symptoms of thiamine deficiency were attenuated and their appearance delayed when pantothenate also was deleted from the diet.

Introduction

Evidence is steadily forthcoming suggesting that the autonomic impulse generation of the heart might depend on an intrinsic synthesis of acetyl choline (ACh) (2–8, 29, 30). It is, therefore, legitimate to assume that an impaired choline acetylation might be the underlying biochemical mechanism of certain disorders of cardiac automatism. Choline acetylation depends on the co-ordinated activity of two multienzyme systems: the donor system and the acceptor system. The former produces the acetyl radical. The latter consists of the apoenzyme of choline acetylation and its chief coenzyme, Lipmann's CoA (cf. 25).

Impairment of choline acetylation, as a cause of disorders of cardiac automatism, can be the consequence of a failure in either the acetyl donor system or the acceptor system.

In the acetyl donor system cocarboxylase (cf. 26), in the acceptor system Lippmann's CoA (cf. 25) play important roles. Since cocarboxylase is a thiamine, CoA, a pantothenic acid derivative, it can be assumed that in nutritional deficiencies of these two vitamins choline acetylation will be impaired and this impairment will cause disorders in cardiac automatism.

It was proved that the thiamine pyrophosphate content diminishes in the hearts of rats on a thiamine-deficient diet (15). It was also shown that the CoA concentration is reduced on a pantothenic-acid-deficient diet (27).

Cardiac disorders have been described in both of these dietary deficiencies. The evidence of their presence in pantothenic acid deficiency is, however, unsatisfactory (28, 31).

It is to be noted that both groups of authors emphasize the suddenness of the onset of symptoms and (in the rat) the irregularity of their appearance.

[1] *Manuscript received March 2, 1955.*
Contribution from the Department of Physiology, University of Ottawa, Ottawa, Canada.
This work was carried out with the aid of grants from the National Research Council of Canada.

In subsequent papers that have described pantothenic acid deficiency, no mention of cardiac disorders has been made. The lack of positive information applies to the other symptoms as well. Most of the research is now directed to the behavior of the pituitary–adrenal system (11, 12, 34, 35) in the pantothenate-deficient animal under stress.

Thus it remains undecided whether cardiac disorders and other symptoms occur when pantothenic acid is the only missing factor from the diet.

In thiamine deficiency, disorders of impulse production and conduction together with other cardiac changes have been described in all species (cf. 18). Thiamine deficiency might cause impaired choline acetylation in two ways: either by a diminution in the cocarboxylase concentration or by structural changes in the heart muscle involving the apoenzyme of choline acetylation. A diminution in the cocarboxylase concentration will act in two ways, by reducing the supply of acetyl precursor from pyruvic acid (26) and that of the energy rich phosphate (14). Quastel *et al.* showed that cocarboxylase increases the rate of formation of ACh in the presence of pyruvic acid (20). Hajdu and McDowall (16) showed that pyruvic acid restores phrenic impulse transmission in the surviving diaphragm by acetyl choline liberation after transmission was stopped by exhaustive stimulation in a glucose-free Krebs' solution.

The heart undergoes great structural changes in complex vitamin B deficiencies which manifest themselves in the weight (19, 23, 24), histological picture (32), and chemical composition (33) of that organ. These observations however were made with diets of complex B vitamin deficiency. It remained therefore to be seen whether such changes occur when thiamine is the only missing factor. It appeared possible that these changes involve the choline acetylating enzyme as well.

The choline-acetylating power of extracts of the acetone powder of heart was first estimated by Comline (9). It is more important that Bülbring and Burn's (4) paper suggested that the apoenzyme may be a very sensitive protein, or its extractability variable. These researches, however, were done with the method of Feldberg and Mann (13*a*) before Balfour and Hebb demonstrated the dependence of choline acetylation on the concentration of CoA (1) and before the possibility existed of adding known amounts of CoA, in safe excess, to the apoenzyme. It is, therefore, possible that in some of these pioneer works the differences in ACh synthesis observed were due rather to differences in the CoA content of the incubated system than to those in the apoenzyme concentration of the heart powders themselves.

In these conditions it seemed necessary to investigate the questions whether the normal choline esterase activity of extracts of the acetone powder of hearts changed in animals on diets deficient only in thiamine, pantothenic acid, or thiamine plus pantothenic acid, and whether the same e.c.g. changes occur on these diets as were described in complex deficiencies.

Experimental

Methods

The male, Wistar strain, albino rats were kept on the experimental diet during the period of May to October 1954. Each of the groups lived in a separate, large, double bottom, screened cage.

At frequent intervals they were weighed and their e.c.g. was recorded. We killed an animal when it ceased to grow, lost weight for some time, the changes in its e.c.g. kept on deteriorating, or when the animal was in a prostated condition.

The diets, supplied by the Nutritional Biochemical Corporation, Cleveland, Ohio, had the following composition: Vitamin Test Casein, 18%; sucrose, 68%; vegetable oil, 10%; U.S.P. Salt Mixture No. 2, 4%. The vitamin fortification mixture is shown in Table I. Tap water was given to drink.

TABLE I

VITAMINS SUPPLEMENTED TO THE NORMAL DIET

Vitamin	Amount per 100 lb. diet	Vitamin	Amount per 100 lb. diet
A (concentrate 200.000 U./gm.)	4.5 gm.	Niacin	4.5 gm.
D (concentrate 400.000 U./gm.)	0.25 "	Riboflavin	1.0 "
Alpha tocopherol	5.0 "	Pyridoxine-HCl	1.0 "
Ascorbic acid	45.0 "	Thiamine-HCl	1.0 "
Inositol	5.0 "	Ca-pantothenate	3.0 "
Choline chloride	75.0 "	Biotin	20 mgm.
Menadione	2.25 "	Folic acid	90 "
P Aminobenzoic acid	5.0 "	Vitamin B_{12}	1.35 "

The normal group (N) was made up of 32 rats and the groups deficient in thiamine (−T) of 12, in pantothenic acid (−P) of 15, and in thiamine plus pantothenate (−TP) of 15 rats each.

The e.c.g., only in lead II., was recorded with a Sanborn Viso Cardiette instrument, the animal being under light ether anesthesia.

In the determination of the choline acetylating activity of the extracts of the acetone dried powder of the heart (A.P.H.), the procedure described by Feldberg and Mann (13*a*, 13*b*) and modified by Balfour and Hebb (1) was used with small modifications. The most important of these was that the CoA was added in known surplus, which we determined to be 33 L.U. per ml. incubate. The increased amount of ACh in the incubate after an hour's incubation was determined by bio-assay on the eserine sensitized frog rectus abdominis.

The entire procedure was standardized as follows:

E.c.g. and body weight were recorded shortly before an animal was killed.

Determinations were made simultaneously on batches of two to three deficient and a similar number of normal animals. In the preparation of the acetone powder of the heart the technic of Bülbring and Burn (4) was used with minor changes.

To the extract—usually 1 ml.—an equal volume of "salt–buffer solution" was added. The pH of the incubate was adjusted to 7.2. The composition of the salt–buffer solution was such as to bring the concentrations of the incubate when diluted with equal volumes of the extract to the values shown in Table II.

TABLE II

COMPOSITION OF THE INCUBATE

Salt	Mgm./ml.	*M*	Salt	Mgm./ml.	*M*
KCl	2.70	0.05	Eserine	0.02	0.00008
$MgCl_2$	0.34	0.005	Choline	2.5	0.018
NaF	0.61	0.015	ATP	3.1	0.005
Phosphate buffer	—	0.006	CoA	0.11	33 L.U.

Of the inorganic constituents 50 ml. was prepared at a time. The organic constituents were weighed freshly for each batch of incubations and dissolved in the salt–buffer mixture: eserine salicylate, choline chloride Merck, ATP disodium salt (99% ATP) chromatographed, CoA (70–75%). The latter compounds were products of Nutritional Biochemical Corporation, Cleveland, Ohio, and were chemically and enzymatically assayed.

Two incubations were made with every extract, one without, the other with citrate, of which 38 mgm./ml. was used. A non-incubated sample was at once acidified, boiled in a water bath, diluted 1 : 12 with frog-ringer, centrifuged, poured off, and stored in a deep freeze till next day when it was assayed with the incubate samples.

The incubations were carried out in air, in Thunberg tubes suspended in a Warburg water bath (37.5° C.), and shaken 140–160 times per minute for one hour. The tubes then were treated as were the non-incubated ones.

Before the assay on the eserinized rectus the pH of the tubes was adjusted to 7.4. The known dilutions of ACh were made with the non-incubated mixture.

Results

Growth and Mortality

Fig. 1 shows the average body weights in the four groups. The weight growth of our −P animals was similar to that of the normal group though the former tended to grow more slowly after about 60 days on the diet.

The −T rats stopped weight-increase after about the 40th day on the diet.

The −TP rats continued to grow till the 57th day, 17 days longer than the ones on the diet deficient only in thiamine, but from that day on, the loss of weight was similar in the two groups. The deletion of pantothenate from the diet already deficient in thiamine diminished the growth retarding and body wasting effect of a pure thiamine deficiency.

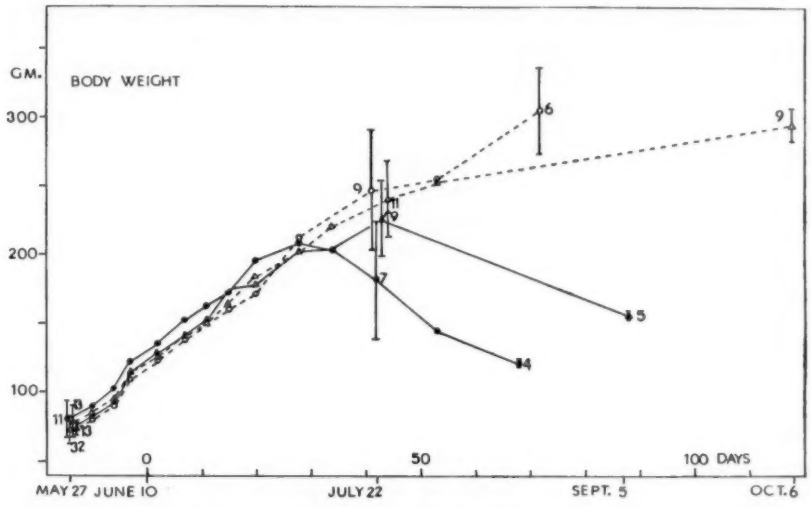


FIG. 1. Body weight changes in rats on complete synthetic diets ○ — — ○ and diets deficient in thiamine ●————●, pantothenic acid △ — — — △, thiamine and pantothenic acid ▲————▲. Vertical lines twice the standard deviation of the averages. Numbers in the figure represent the number of rats alive at that point. Lower abscissa chronological scale, upper abscissa days on the experimental diet.

Other Symptoms

We saw none of the −P deficiency symptoms described in the literature.

The thiamine deficient animals showed the following symptoms: torpidity, curved back, avoidance of movement, gingerly gait, slow, spastic steps suggesting painful joints, loss of hair, reddish hyperemic dry skin, loss of appetite. Their response to stimuli was unpredictable. The rat might seem to be quite unsensitive to noise or to a pinch of the tail or it might respond to the same stimulus with a high jump. Death followed a lethargic state lasting many hours.

The symptoms of the −TP animals were the same as those of the −T group except that they were attenuated and retarded.

The death rates can be seen in Fig. 2. Up to July 13, only spontaneous deaths occurred. From this date on animals were also killed.

A comparison of the death rates of the three deficiency groups shows that the lowest spontaneous death rate occurred in the −P group, and the largest in the −T. The −TP group, as in the case of the body weight curve, lies in between the two other groups but nearer to the −T.

The normal rats showed the greatest death rate. Their curve is shown only till June 30 because from that time on different numbers of animals were killed for the parallel determinations. The growth rate of the normal rats was satisfactory. There was no diarrhea. The behavior and skin of the animals showed no abnormality. There was considerable cannibalism amongst

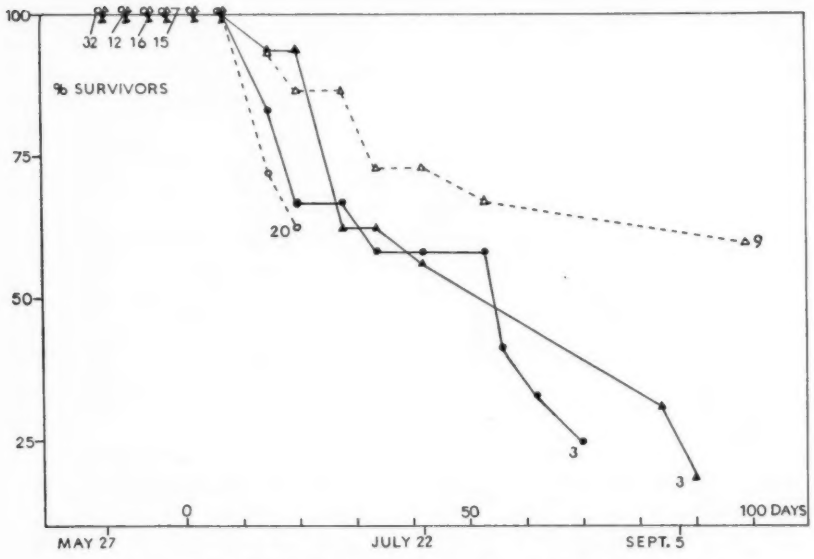


FIG. 2. Death rate in the four groups of rats. Symbols as in Fig. 1.

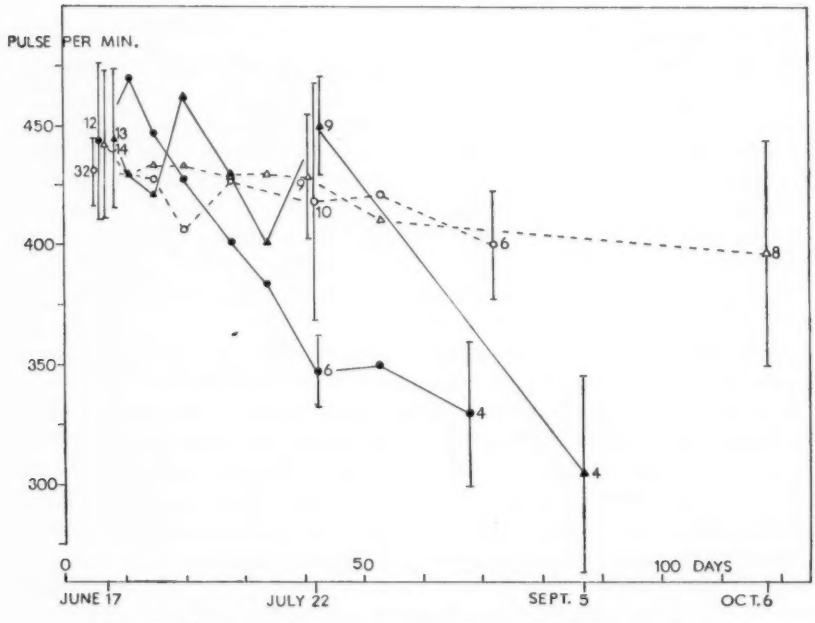


FIG. 3. Heart rates in the four groups of rats. Symbols as in Fig. 1.

them. In those cases in which a post-mortem could be carried out on the remains no pathological change could be seen. As the summer was very hot we attributed these deaths to the heat, and cannibalism.

Electrocardiographic Observations

We observed five properties of the e.c.g.: rate, height of R and T waves, P-R time, and irregularities of the pattern.

Fig. 3 shows the averages of the *heart rates* in the four groups plotted against time.

Here, as in the cases of body weight and mortality, the four groups fall into two subdivisions: the normal and −P animals forming the one and the −T and −TP the other. In the former subdivision both groups have a tendency to slow down; they do so steadily and slightly.

The −T and −TP animals after a period of fluctuation become bradycardic, as first described by Drury, Harris, and Maudsley (10). The period of fluctuation is short, 20 days, in the −T animals, longer, 50 days, in the −TP group. The prolongation of the period of fluctuation in the −TP animals is another manifestation of the attenuating effect of the deletion of pantothenic acid from the thiamine-deficient diet. The reduction in the heart rate in thiamine deficiency is not the consequence of the appearance of exceptionally low heart rates, never occurring in normal rats; it is due to the gradual disappearance of high heart rates occurring frequently while the animal is normal.

Calculations showed that there was no correlation between the following parameters: (1) initial heart rate and decrement in heart rate except in the −T group in which r was found to be -0.74, $0.02 < P < 0.05$, D.F. $= 6$, (2) initial heart rate and length of life, (3) heart rate at the day of death and time on the deficiency diet.

Fig. 4 shows the averages of the voltage of the R wave in the four groups. Here as in the case of the heart rates, the normal and −P animals form one subdivision the −T and −TP another. In the normal and −P animals the voltage rises slowly and not much. In the −T and −TP animals after an initial period of small fluctuations it rises rapidly and to high values.

The increase of the R voltage in a—probably complex—vitamin B deficiency was first described by van Heerswynghels and Thomas (33). The rise starts somewhat sooner, is faster, and attains a higher value in the −T group than in the −TP.

Calculations of correlation coefficients revealed no significance of correlation between height of R voltage and heart rate in the normal group calculated for the entire period, nor in the deficient groups during the initial phase on the deficiency diets as long as the changes were not marked (Table III). The value of $-r$ rose, however, in all the three deficiency groups during the second phase, when the changes became marked.

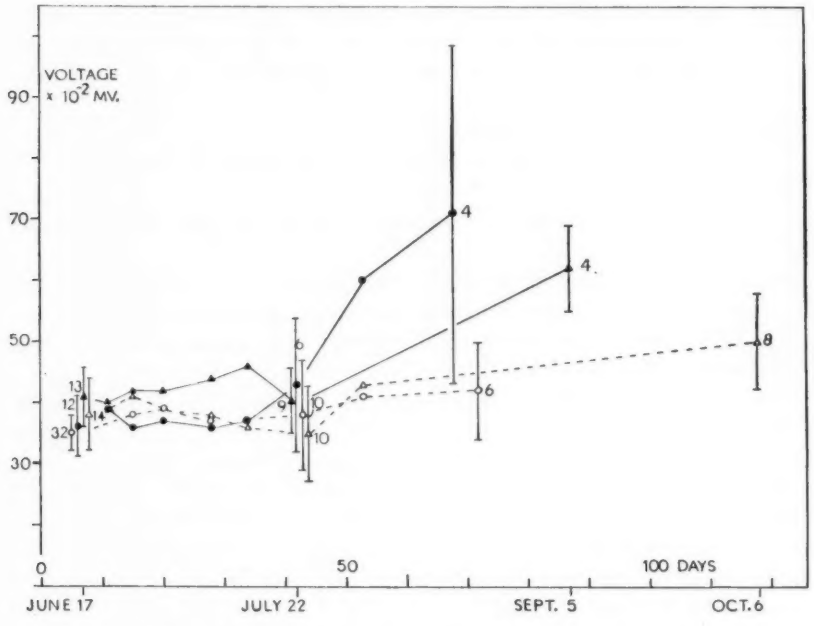


FIG. 4. Voltage of the *R* wave in the four groups of rats. Symbols as in Fig. 1.

TABLE III

CALCULATIONS OF CORRELATION BETWEEN HEART RATE AND *R* VOLTAGE DURING DIFFERENT PERIODS ON THE FOUR EXPERIMENTAL DIETS

Groups	In normal state			After 44 days on def. diet		
	r	*n**	*P*	*r*	*n*	*P*
N	−0.09	71	≫0.10	—	—	—
−T	−0.05	49	≫0.10	−0.29	25	>0.10
−TP	−0.10	76	≫0.10	−0.66	13	0.02
−P	−0.07	14	≫0.10	−0.41	27	0.05

* *n = number of e.c.g. taken during the experimental period under consideration.*

The value of *P* also tended towards significance in all the three deficiency groups. It reached the level of 0.02 in the −TP group, 0.05 in the −P, but only 0.1 in the −T animals. We think this is the consequence of the fact that in each group death reduced the original number of animals and that these few animals lived longer on the diets. The longer an ever decreasing number of animals lives on the deficient diet the greater is the likelihood that both bradycardia as well as increase in the *R* voltage will develop.

This assumption is borne out by the fact that the increase in the P value is directly proportional to the length of time on the deficient diets and inversely to the number of animals.

As will be seen from Fig. 5 there is a slight trend to a prolongation in the P-Q duration. The averages show great fluctuation in all the four groups. Contrary to previous authors who described an increase in P-Q duration in complex vitamin B deficiencies, we did not find a difference in this trend in the four groups. The discrepancy is probably due to the circumstance that the previous authors did not follow the changes in animals on a normal diet and in those on a deficiency diet throughout the entire experimental period and they had no measure of the variability.

In the course of the work we have noted that the abnormalities in the e.c.g. described by earlier workers in vitamin B deficiency occur also in normal animals, though less frequently and to a smaller degree. Thus, to evaluate the effect of the deficiencies a statistical approach became necessary.

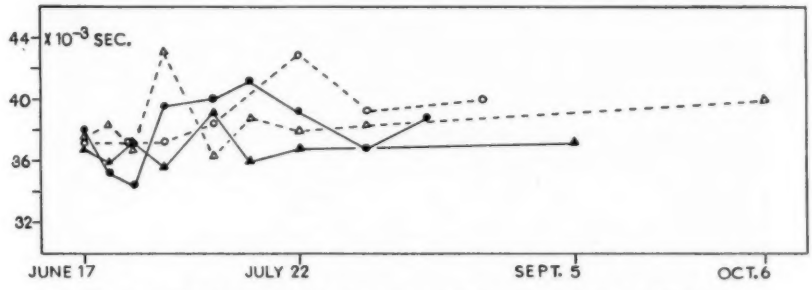


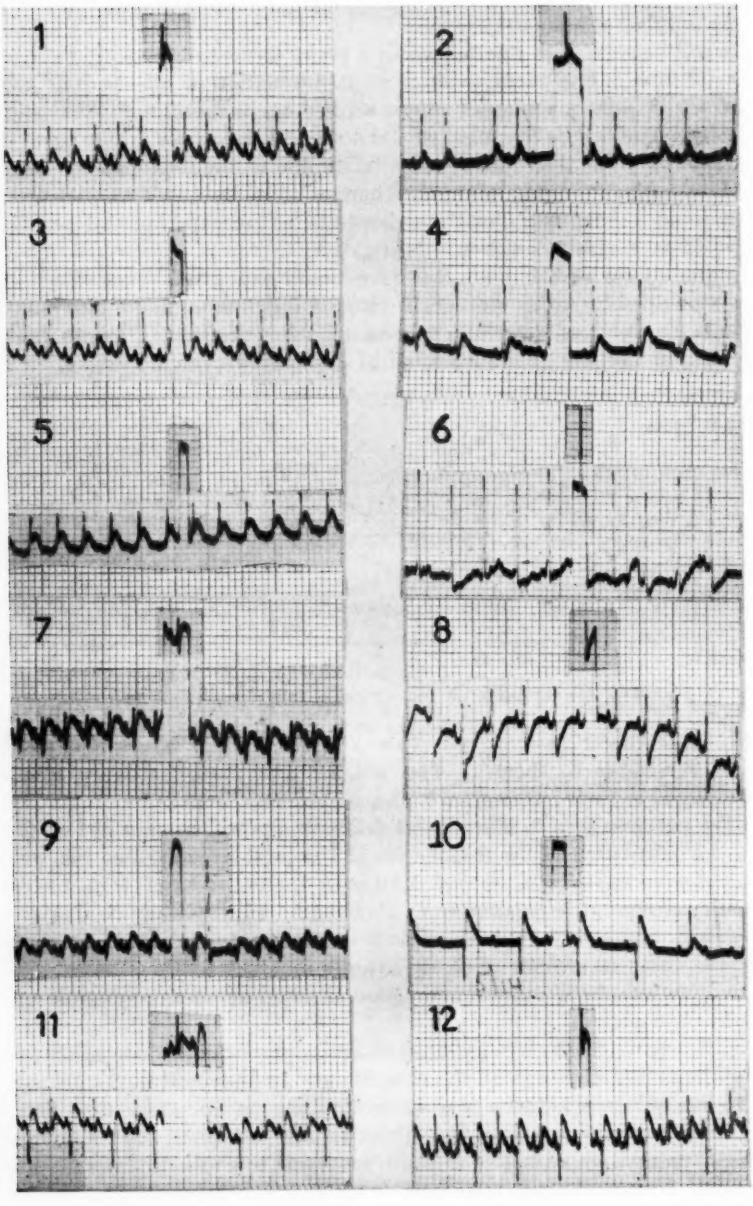
FIG. 5. P-Q duration in the four groups of rats. Symbols as in Fig. 1.

We observed certain patterns of the P, QRS, and T waves and ascribed arbitrary weights to them. The weights given were roughly inversely proportional to their frequencies in the normal population.

A few patterns seen in normal and deficient rats will be seen in Fig. 6.

In the calculation of the frequencies of the total irregularities the weights ascribed were as follows: normal pattern of the entire e.c.g. = 1, uncertain normalcy of the entire pattern = 2; P changes: slightly wavy or absent = 3, wavy = 5, very wavy = 7; deviations in QRS = 9–11, absent or negative T = 13. The occurrence of these patterns was counted in all four groups, the weights attached, and the averages calculated at several intervals of the experimental period.

Fig. 7 shows that the four groups fall again into the same two subdivisions, the −T and −TP animals in the one, the −P and normals in the other. In the former subdivision, after about the 40th day on the deficiency diet, the frequency of occurrence of irregularities rapidly increases. In the normal and −P animals there is also a slight tendency towards an increase in the frequency of irregularities and this tendency is somewhat greater in the −P animals.


1
2
3
4
5
6
7
8
9
10
11
12

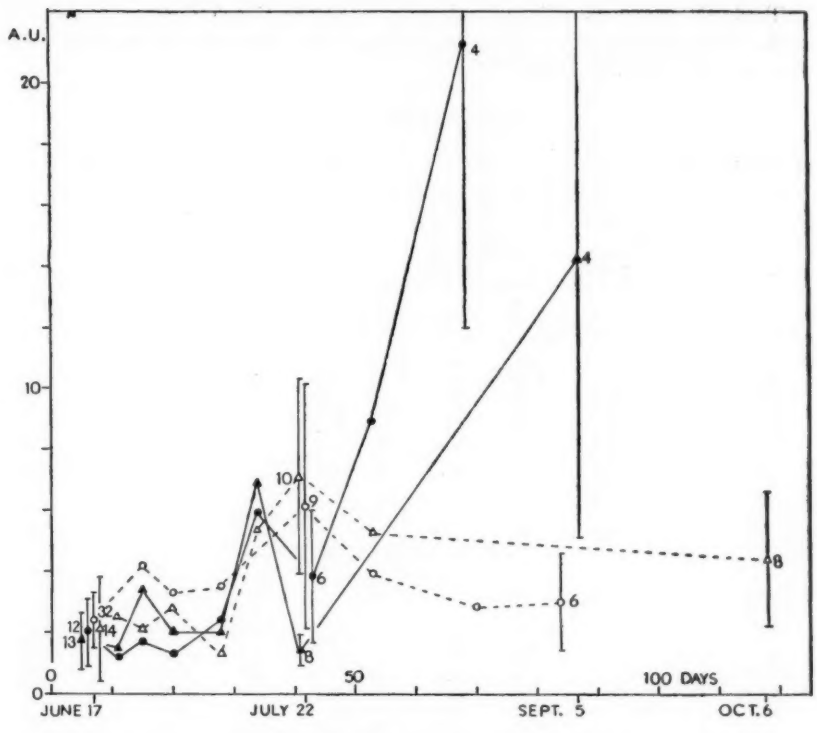


FIG. 7. Frequency of irregularities in the pattern of e.c.g. in the four groups of rats. A.U. = arbitrary units; for details see text. Symbols as in Fig. 1.

FIG. 6. Patterns of e.c.g. in normal rats, left column; and in rats on deficient diets, right column. 1. "Ideal" pattern, most frequently seen in normal rats. 2. −TP rat No. 29, 20 days on the diet, incomplete auriculoventricular block. *R* voltage well within 1 σ of the normal variation. 3. Normal rat with *R* wave higher than 1 σ of the normal voltage and with slurs. 4. −TP rat No. 19, 90 days on diet, bradycardia, increased *R* voltage, auricular flutter. 5. Normal rat, no or very low *P* wave. 6. −T rat No. 44, 70 days on diet, slight bradycardia just near to 1 σ of the normal, auricular fibrillation, low *S* wave, absence of *P* and *T* waves. 7. Normal rat, longer than average *P-Q* duration, low *R* voltage, negative *S*, prolonged *T*. 8. −T rat No. 73, 70 days on diet, bradycardia just outside 1 σ, *R* voltage normal, strongly negative *S*, no *T* wave. 9. Normal rat, no *R* wave except one large slurred. 10. −TP rat No. 19, 34 days on diet, slight auricular bradycardia, dissociation of auricular and ventricular electrical systoles, ventricular bradycardia, mostly negative *R* wave (?), but for one complete impulse with normal pattern. 11 and 12. Rat No. 46, showing that an individual pattern of the e.c.g. is retained throughout life and is not altered by a pantothenic-acid-deficient diet. Both on normal diet (11) and 99 days on the deficient diet, normal rate, normal shapes of *P*, and *T* waves, but every other *R* wave is negative. The high deflections in the middle are the voltage calibrations = 1 mv.

Table IV shows the voltage and duration of the *R* and *T* waves in the −T and −TP animals before they were put on the deficient rations and when the changes fully developed.

TABLE IV

VOLTAGE AND DURATION OF THE *R* AND *T* WAVES IN THE −T AND −TP ANIMALS

	Before deficiency				During deficiency			
	R wave		*T* wave		*R* wave		*T* wave	
No.	mv.	10^{-3} sec.	mv.	10^{-3} sec.	mv.	10^{-3} sec.	mv.	10^{-3} sec.
				−T animals				
1	0.35	8	0.21	60	0.85	16	0.21	60
39	0.50	12	0.28	60	0.64	12	0.28	100
44	0.57	8	0.25	60	1.13	12	0 or inverse	0
55	0.50	8	0.28	80	0.78	8	0.21	80
64	0.57	8	0.21	80	0.71	4	0	0
73	0.43	8	0.28	80	0.43	8	0	0
74	0.43	8	0.21	80	0.92	8	0.32	80
$\bar{x}$ =	0.48	9	0.25	71	0.78	10	0.15	46
n =	7		7		7		7	
σx =	0.09	0.8	0.01	2.4	0.71	0.4	0.28	56
				−TP animals				
V7	0.42	16	0.17	60	0.62	8	0.41	100
18	0.56	8	0.28	60	0.48	8	0.27	60
19	0.34	8	0.21	60	0.75	12	0.27	100
34	0.49	8	0.28	60	0.55	8	0.27	80
50	0.34	8	0.21	60	0.55	4	0.34	60
68	0.70	12	0.21	40	0.82	12	0.27	60
79	0.49	4	0.21	60	0.68	12	0	0
82	0.49	8	0.28	40	0.74	8	0.34	80
$\bar{x}$ =	0.48	9	0.23	63	0.65	9	0.27	77
n =	8		8		8		8	
σx =	0.19	0.4	0.02	2	0.22	0.4	0.21	24

Van Heerswynghels and Thomas (33) found an increase in the *T* voltage. In our —T animals the most characteristic change is that both the voltage and the duration of the *T* wave become very variable as a comparison of the averages and the standard deviations clearly proves. Out of seven rats, three had no *T* wave or an inverse *T* wave.

The deletion of pantothenate from the thiamine-deficient diet has its attenuating effect in this *T* wave change as well. The great irregularity is diminished, and there is a small tendency to an increase both in voltage and duration.

Choline Acetylase Activity

Table V shows the results of the determinations of enzyme activity and cardiac rates. The results are so arranged that the broader horizontal spaces in the table divide the batches determined by the same worker on the same day.

TABLE V

CHOLINE ACETYLASE ACTIVITY, HEART RATE, HEART WEIGHT, BODY WEIGHT, AND TIME ON THE EXPERIMENTAL DIETS IN THE FOUR GROUPS OF RATS

No.	Days on diet	Heart rate	ACh synthesis μgm./gm. hr.		Body wt., gm.	Heart wt., mgm.
			No citr.	With citr.		
			Thiamine-deficient rats			
67	28	320	94	—	125	752
3	N*	—	80	—	202	775
63	N	420	80	—	223	834
39	54	265	36	112	115	408
55	54	315	32	36	118	436
6	N	420	26	35	270	916
16	N	405	32	35	200	823
44	70	310	30	36	123	651
73	70	330	23	36	124	637
21	N	410	23	36	296	916
15	N	355	30	36	310	906
64	61	305	20	—	118	466
1	56	375	20	—	125	484
74	70	375	8	29	115	578
12	N	435	8	58	284	922
14	N	375	8	29	284	883
			Pantothenate-deficient rats			
80	99	—	34	24	270	933
65	99	440	35	36	250	825
46	99	470	36	108	266	992
4	N	360	35	36	346	1513
20	123	270	72	130	314	1015
48	123	435	101	130	368	995
71	123	360	72	130	325	950
41	N	305	72	130	278	848
52	N	—	72	86	217	759
31	126	435	30	162	268	822
73	126	465	36	205	250	832
15	126	360	36	205	334	1080
Y1	N	385	36	205	385	922
Y2	N	330	36	122	330	777
			Thiamine- and pantothenate-deficient rats			
82	93	335	25	155	154	522
79	93	340	25	263	150	529
2	N	420	22	209	348	1127
1	N	440	25	223	220	746
19	99	225	3	33	155	571
20	99	315	3	79	156	532
34	99	—	14	21	160	770
s2	N	300	18	36	250	715
3	N	355	18	30	292	843
68	32	470	79	—	178	803
62	32	410	40	—	160	641
66	N	510	36	—	204	806
69	N	420	130	—	227	727
7	35	410	151	—	115	489
8	N	420	136	—	120	520
5	N	410	146	—	122	461
4	N	450	137	—	111	394

Rats — TP68 and N66 had an inflamed leg.
Rat — TP62 had pneumonia.
Rat — TP7 had peritonitis.
* *N = normals.*

It will be seen that the values spread from 8 to 151 μgm. ACh/gm. hr. acetone powder, without citrate and from 21 to 263 μgm. with citrate. We believe that the values below 20 μgm. are due to some accidental difference in the procedure.

Comline (9) obtained synthesis up to 90 μgm./gm. hr. in auricles and 0 to 25 μgm/gm. hr. in ventricles of hearts (species not given); Bülbring and Burn (4) found in normal rabbit auricles a synthesis of 40 to 75 μgm./gm. hr. Our normal average for whole rat hearts is 55 μgm./gm. hr. with a maximum of 223 μgm./gm. hr., which tallies well with the above findings.

To find an eventual difference in the acetylating power of the different groups as compared to the normal group the same batches should be compared. By doing this it will be seen that there is no difference in this value in any of the groups. Under strictly identical conditions extracts having the same activities could be obtained from deficient hearts as from normal ones. An analysis of variance between groups 2 and 3, within groups 1 and 2, and total 1 and 3 confirmed this conclusion both for the choline acetylating activity without and with addition of citrate. The variance ratio in the "with" group was $i = 0.63$, $n_1 = 3$, $n_2 = 30$; in "without" group $i = 0.64$, $n_1 = 3$, $n_2 = 43$. Significance level in both groups is below 20%.

No correlation was found either between the enzymic acitivity of the extracts and heart rate or between the enzymic activity and heart weight or time on the deficient diets in any of the four groups.

Discussion

We found that the extracts of the acetone powder of thiamine deficient bradycardic hearts acetylate choline at the same rate as do normal hearts if CoA and ATP are added in excess.

Nachmansohn and Wilson (22) expressed the view that the extraction in the method of Feldberg and Mann, i.e. the method used in this work, may not be adequate for the extraction of the choline acetylating system from all tissues. This objection to our results cannot be made because the extracts obtained from normal and deficiency hearts had the same activities. Thiamine deficiency, therefore, did not change the extractability of the apoenzyme system.

The method in its present form, however, is not suitable for the determination of the concentration of the choline acetylating apoenzyme. However it appears likely that the concentration of the enzyme remained also unchanged in the deficiency hearts because extractability depends also on concentration. Though numerous and great changes take place in the biochemical and microscopical structure of the heart cells in thiamine deficiency, they do not involve the choline acetylating apoenzyme.

It was pointed out in the Introduction that a diminution in the cocarboxylase concentration may also lead to a diminished choline acetylation. The next stage in the study of the biochemistry of the disturbance of cardiac

automatism in thiamine deficiency may be a correlative determination of acetyl choline release, cocarboxylase activity, and disturbance in cardiac automatism in thiamine-deficient hearts.

To the question whether there are changes in the cardiac automatism on a diet deficient in thiamine alone, we obtained an affirmative reply. The conclusion is justified that the changes in e.c.g. described in rats on a multi-deficient diet were due to the absence of thiamine itself.

There are two circumstances that seem to us noteworthy: (1) Differences from the "ideal" e.c.g. occur in normal as well as in deficient rats but in them they are less marked and less frequent. (2) Occurrence of death and frequency of irregularities of cardiac impulse generation and propagation are not correlated. Death occurs in animals whose e.c.g. is almost normal; on the other hand irregularities (especially those of the sinoatrial complex) may start early, may become more and more frequent, manyfold, and severe yet the animal survives for weeks. No doubt the explanation of this is that death occurs in thiamine deficiency not only as a consequence of heart failure, and that there is no correlation between e.c.g. and cardiac viability.

To the question whether a singular pantothenic acid deficiency affects the e.c.g., we obtained a negative reply.

As regards the possible explanation of the discrepancy between the symptom rich picture of pantothenic acid deficiency in the rat described by earlier authors and the symptom poor findings of the more recent workers (including ourselves) it appears that the chief difference in the experimental conditions of the two groups of workers is that the earlier workers fed a multideficient diet, the latter a uni-deficient diet*.

Thus Supplee *et al.* (31), who first described sudden death and cardiac hypertrophy with auricular hemorrhage, supplemented their basic casein–sucrose diet with thiamine, riboflavin, and pyridoxine only. Olson and Caplan (10), to whom we owe the knowledge of a diminution in the CoA content on a pantothenic-acid-deficient diet, added choline also to the above supplements.

As in the hearts of our rats on the pantothenic-acid-deficient diet there was probably no great diminution in the CoA content, no disorder in their automatism was to be expected. This was indeed the case. The question still remains unanswered whether a disorder develops when a diminution in the CoA content is produced.

The absence of symptoms on pantothenic-acid-deficient diets is sometimes attributed to a synthesis by the intestinal flora. In this connection there are two aspects to which attention has not yet been paid.

One is that in the early days of pantothenic acid research, few or no precautions were taken against refection, yet the symptom rich state developed, the diets being multideficient.

* *The casein used by us contained only 0.15 μgm./gm. pantothenic acid (informatios of the manufacturer).*

The other is that in our experiments thiamine deficiency did develop although the precautions against bacterial synthesis and refection were the same for those rats as for the pantothenic-acid-deficient ones.

Two admissions seem to be justified: (1) if it is bacterial synthesis that provides the rat with pantothenic acid it takes place more easily on a complete than on a multideficient diet; and (2) that pantothenic acid is synthesized more easily than thiamine.

We obtained a somewhat unexpected reply to the question of what is the effect of the simultaneous absence of thiamine and pantothenic acid on cardiac automatism and on other deficiency symptoms. We expected an enhancement of the thiamine deficiency symptoms because we thought that to the diminution in the utilization of pyruvic acid caused by a lack of cocarboxylase the diminution caused by a lack of CoA would be added. Contrary to this both the bradycardia as well as the other symptoms were delayed.

Morgan and Lewis (21) observed another similar delaying action of a lack of pantothenic acid in the diet. These authors found that absence of pantothenic acid prevented the development of fatty liver otherwise seen on a diet deficient only in choline.

The delaying action of pantothenic acid deficiency may be a cocarboxylase sparing action.

Acknowledgments

We have pleasure in expressing our appreciation of the repeated discussions we have had with Dr. Margaret Beznák during this work and the preparation of this paper.

Our thanks are due to Mr. F. X. Fournier and Mrs. M. T. Fournier for competent technical help.

References

1. Balfour W. E. and Hebb, C. J. Physiol. 118 : 94. 1952.
2. Briscoe, S. and Burn, J. H. J. Physiol. 126 : 181. 1954.
3. Briscoe, S. and Burn, J. H. Brit. J. Pharmacol. 9 : 42. 1954.
4. Bülbring, E. and Burn, J. H. J. Physiol. 108 : 508. 1949.
5. Bülbring, E., Burn, J. H., and Kottegoda, S. R. J. Physiol. 118 : 31*P*. 1952.
6. Burn, J. H. and Kottegoda, S. R. J. Physiol. 121 : 360. 1953.
7. Burn, J. H. and Walker, J. M. J. Physiol. 124 : 489. 1954.
8. Burn, J. H. and Wane, J. R. J. Physiol. 108 : 104. 1949.
9. Comline, R. S. J. Physiol. 105 : 6*P*. 1946.
10. Drury, A. N., Harris, L. J., and Maudsley, C. Biochem. J. 24 : 1632. 1930.
11. Ershoff, B. J. Nutrition, 49 : 373. 1953.
12. Ershoff, H. H., Alfin-Slater, R., and Gaines, J. J. Nutrition, 50 : 299. 1953.
13. Feldberg, W. and Mann, T. J. Physiol. (*a*) 104 : 8. 1945. (*b*) 104 : 411. 1946.
14. Green, D. E. Science, 115 : 661. 1952.
15. Gruber, M. Biochim. et Biophys. Acta, 9 : 333. 1952.
16. Hajdu, J. and McDowall, R. J. S. J. Physiol. 108 : 502. 1949.
17. Kossmann, C. H. *In* Disorders of the circulatory system. The MacMillan Co., New York. 1952. p. 150.

18. LEPESCHKIN, E. Modern electrocardiography. Vol. I. Williams & Wilkins Company, Baltimore. 1951. p. 618.
19. MCCARRISON, R. Indian J. Med. Research, 6 : 275. 1919.
20. MANN, P. J. G. and QUASTEL, J. H. Nature, 145 : 951. 1941.
21. MORGAN, A. F. and LEWIS, E. M. J. Biol. Chem. 200 : 839. 1953.
22. NACHMANSOHN, D. and WILSON, I. B. Advances in Enzymol. 12 : 259. 1951.
23. NAKAMURA, K. and MURATA, M. Trans. Japan. Pathol. Soc. 18 : 177. 1928.
24. NEWCOMBE, C. Indian J. Med. Research, 17 : 721. 1930.
25. NOVELLI, G. D. Physiol. Revs. 33 : 525. 1953.
26. OCHOA, S. Physiol. Revs. 31 : 56. 1951.
27. OLSON, T. S. and KAPLAN, N. O. J. Biol. Chem. 175 : 515. 1948.
28. SCHAEFER, A. E., MCKIBBIN, J. M., and ELVEHJEM, C. A. J. Biol. Chem. 143 : 321. 1942.
29. SPADOLINI, I. Arch. fisiol. 52 : 443. 1953.
30. SPADOLINI, I. and GIACHIETTI, D. Arch. fisiol. 52 : 329. 1953.
31. SUPPLEE, G. C., BENDER, R. C., and KAHLENBERG, O. J. Endocrinology, 30 : 355. 1942.
32. THOMAS, R. M., MYLON, E., and WINTERNITZ, M. C. Yale J. Biol. and Med. 12 : 345. 1939.
33. VAN HEERSWYNGHELS, J. and THOMAS, J. Cardiologia, 9 : 211. 1945.
34. WINTERS, R. W., SCHULTZ, R. B., and KREHL, W. A. Endocrinology, 50 : 377. 1952.
35. WINTERS, R. W., SCHULTZ, R. B., and KREHL, W. A. Endocrinology, 50 : 388. 1952.

THE METABOLIC EFFECTS OF INTRAVENOUSLY ADMINISTERED ALDOSTERONE IN A TOTALLY ADRENALECTOMIZED HUMAN[1]

By J. C. Beck[2], C. P. Giroud, I. Dyrenfurth, and E. H. Venning

Abstract

Six hundred micrograms of aldosterone administered intravenously to a totally adrenalectomized patient caused water retention and weight gain without apparent alteration in the sodium and potassium balance. A marked increase in the urinary nitrogen excretion occurred during the aldosterone infusion.

Observations on the clinical and metabolic effects of aldosterone in man have been limited by the small quantities of this substance which have been available. Ward (17) and his collaborators at the Mayo Clinic have studied its effects in patients with rheumatoid arthritis, while Mach (6), Kekwick and Pawan (4), and Prunty (9) have observed its effects in patients with Addison's disease. All have observed a fall in urinary sodium and chloride and a concomitant rise in urinary potassium excretion. An accompanying water retention has been an inconstant feature. No alteration in protein and carbohydrate metabolism has been demonstrated in normals but in patients with Addison's disease, aldosterone has been observed to normalize both the fasting blood sugar and the flat glucose tolerance curve. In normals there has been no significant change in the eosinophile count, while in the patients with Addison's disease a fall has been reported. There has been no change in the ability of the patient with Addison's disease to handle a water load after aldosterone administration. Aldosterone has not exerted any antirheumatic effect in rheumatoid arthritis but has resulted in clinical and laboratory evidence of improvement in patients with Addison's disease. Its activity in the replacement therapy of patients with Addison's disease is 25 to 30 times that of desoxycorticosterone. Mach (6), Simpson and Tait (12, 13), and Kekwick and Pawan (4) have noted that aldosterone depresses the salivary sodium–potassium ratio both in normals and in patients with Addison's disease, Mach (6) however did not observe any change in the sweat sodium. This present study was carried out concomitantly with investigations by Venning *et al.* (16) on the recovery of aldosterone in the urine following its intravenous administration. It was found that 13.3% of the administered aldosterone was excreted in the urine.

[1] *Manuscript received April 13, 1955.*

Contribution from McGill University Clinic, Royal Victoria Hospital, Montreal, Quebec. Supported by Grants from the National Research Council of Canada; Department of the Army Medical Research and Development Board, Washington, D.C., and from the Lilly Research Laboratories, Indianapolis, Indiana.

[2] *Markle Scholar.*

Clinical Material and Methods

A metabolic balance study was carried out in a 21-year-old female patient who was first admitted to hospital in 1952 with classical Cushing's syndrome. A partial left adrenalectomy was performed on January 30, 1953, and a total right adrenalectomy on November 16, 1953. In July 1954 there was a recurrence of her symptoms and signs and a total left adrenalectomy was carried out on August 16. The weight of the hyperplastic adrenal remnant was 8.88 gm. The postoperative course was uneventful and a satisfactory maintenance dose of 12.5 mgm. of cortisone acetate every eight hours was begun on August 24. This dose was reduced to 7.5 mgm. every 12 hr. on September 11 (eighth day of study) in preparation for the aldosterone infusion, with no apparent ill effect. On the 12th day 600 μgm. of aldosterone dissolved in 1500 cc. of 5% glucose and water was administered slowly intravenously over the total 24 hr. period at an approximate rate of 1 ml. per minute. During this period a comparable amount of carbohydrate was subtracted from the dietary intake. This was part of an extensive metabolic balance study in this patient. The patient was placed on a known dietary intake which was calculated with the aid of food tables (14, 15). The daily values of the dietary constituents varied since three diets of approximately the same value were given in a cyclic fashion in an attempt to reduce the monotony of a prolonged balance study. These variations ranged from: sodium, 80.6–124.1 meq.; potassium, 92.2–107.4 meq.; protein, 100.9–103.7 gm.; fat, 74.9–80.0 gm.; carbohydrate, 199.2–200.6 gm.; total calories, 1941–1981 and ascorbic acid, 372.1–378.5 mgm. Complete 24-hr. urine specimens were collected for this purpose.

Nitrogen balance studies were conducted employing a factor of 1.3 gm. per 24 hr. for the fecal nitrogen. Ascorbic acid balance studies were carried out as previously described (1). Electrolyte studies consisted of serum and urinary electrolyte determination with the exception of the calcium and phosphorus in which no balance study was carried out but only the urinary levels determined. Salivary sodium and potassium was measured in the post-absorptive state one day prior to, during, and two days after the aldosterone infusion. To stimulate salivary flow the subjects chewed paraffin and specimens were collected over two consecutive 10-min. periods. The first 10-min. specimen was discarded since it was not considered to represent a sample of normal salivary secretions. The second specimen was centrifuged and an aliquot made up in distilled water. The following methods were employed: sodium and potassium (flame photometry using an internal lithium standard); ascorbic acid, Roe and Kuether (11); urine nitrogen, Peters and Van Slyke (7); urine creatinine, modification of Folin and Wu (8); urine calcium, Larson and Greenberg (5); urine phosphorus, Fiske and Subbarow (2) and blood sugar, Folin (3).

Results

The metabolic balance data are charted in Figs. 1 and 2. Clinically there was no subjective or objective change in the condition of the patient with the exception of a 2½ lb. weight gain at the conclusion of the aldosterone infusion. This weight was lost in the subsequent 24 hr. There was no alteration in the blood pressure during the infusion. Coincident with the weight gain there was a fluid retention of 1075 ml. followed by a urinary volume of 456 ml. in excess of the intake on the day after the aldosterone infusion. There was no change in the sodium and potassium balance and no significant alteration in the serum sodium, potassium, and CO_2 combining

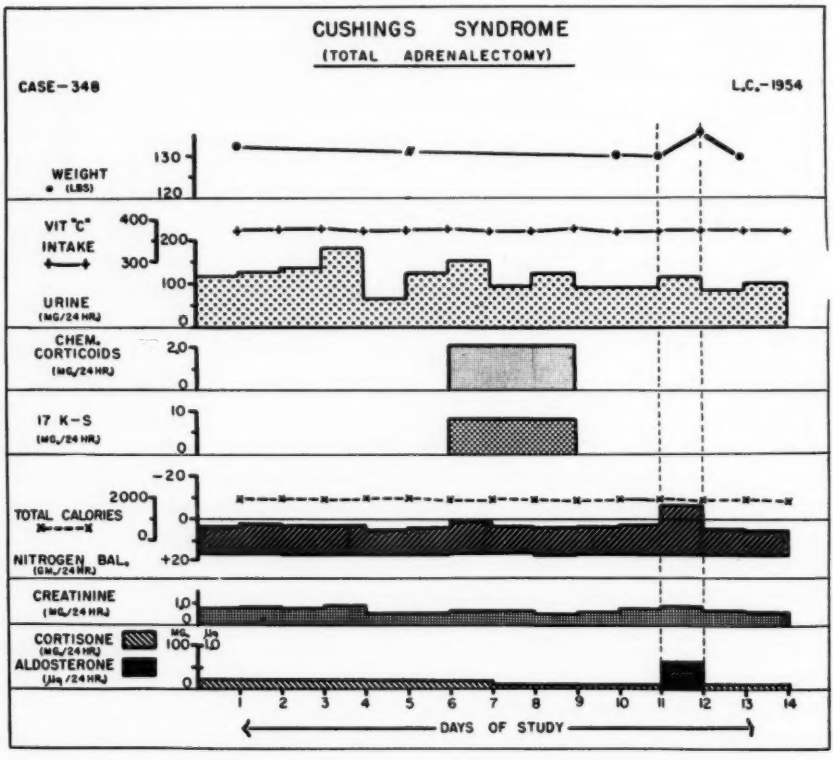


FIG. 1. Metabolic balance data during aldosterone infusion. There is a horizontal base line at zero. The intake is charted downward from this base line; the urinary and fecal excretions are then measured upward from the intake line toward the base line. If the output exceeds the intake, the final level will be above the base line; if the output is less than the intake, the final level will be below the base line. Thus a negative balance is indicated by a shaded area above the base line and a positive balance by a clear area below the base line. This figure shows an increase in weight and a negative nitrogen balance on the day of aldosterone administration.

power. An increase in the urinary calcium excretion was noted during the aldosterone infusion but no significant alteration in urinary phosphorus excretion occurred.

There was no change in the ascorbic acid balance. During the aldosterone infusion there was an increased urinary nitrogen excretion which resulted in a negative balance of 6.9 gm. The nitrogen balance returned to its previous level on cessation of the infusion. There was a rise in the fasting blood sugar, the significance of which is difficult to interpret.

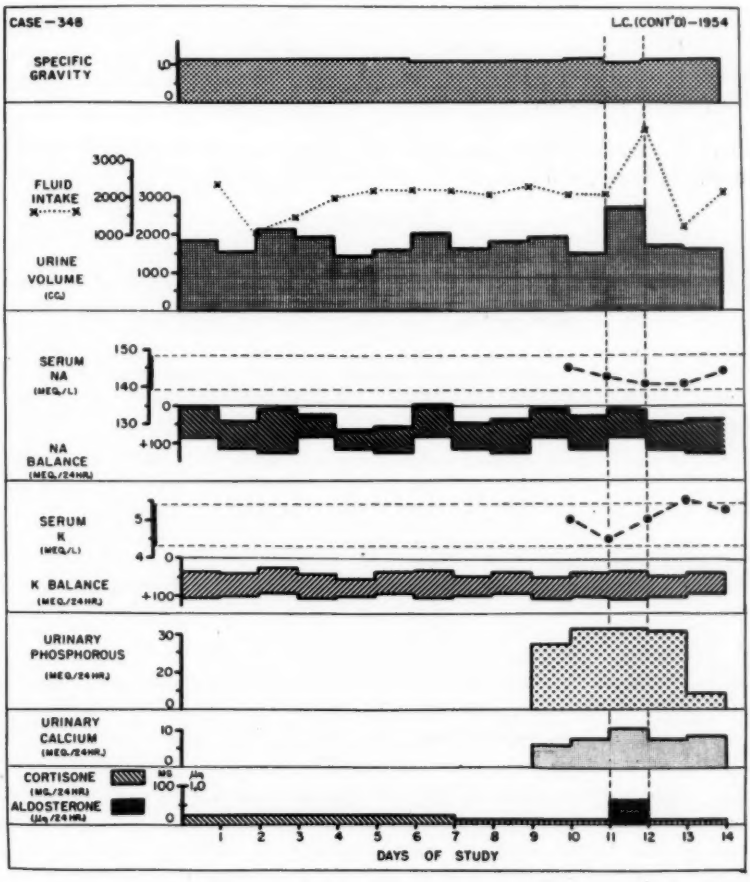


FIG. 2. Metabolic balance data during aldosterone infusion showing alterations in urine volume, sodium and potassium balance and in the urinary excretion of phosphorus and calcium.

The salivary sodium–potassium ratios are presented in Table I. There is a slight decrease in the ratio at noon on the day of the infusion and on the first postinfusion day.

TABLE I

SALIVARY SODIUM–POTASSIUM RATIO

Day of study	8 a.m.	12 noon	3.30 p.m.
11	0.063	0.059	—
12*	0.059	0.035	0.063
13	0.069	0.039	0.054
14	0.063	0.049	—

* *Infusion of aldosterone.*

There was no significant change in the direct eosinophile count or the total white blood count, Table II.

TABLE II

EOSINOPHILE AND TOTAL W.B.C.

	Eosinophiles			White blood cells		
Day of study	9 a.m.	12 noon	4 p.m.	9 a.m.	12 noon	4 p.m.
10	84	31	56	11,825	12,675	12,725
11	71	56	78	10,900	12,100	12,100
12*	78	46	90	11,200	11,450	11,375
13	37	46	56	10,850	11,700	11,700
14	34	21	40	10,900	11,050	11,250

* *Infusion of aldosterone.*
Direct eosinophile counts were determined by the method of Randolph (10).

Discussion

The interpretation of the observations made in a single clinical and metabolic balance study of this nature is difficult but the unavailability of aldosterone has prevented further studies from being made. There are several findings which are at variance with those reported by previous investigators (4, 6, 9, 15). The most striking of these is the water retention and weight gain without apparent alteration in the sodium and potassium balance. This may in part be due to the route of administration of aldosterone, Prunty (9) reporting that the urinary sodium retention decreased during continuous intravenous infusion of aldosterone as compared with its intramuscular administration to an Addisonian patient. The water retention on the day of aldosterone infusion may be associated with an impaired diuretic response to

a water load. However this abnormality was not demonstrated when the patient was on a maintenance dose of 37.5 mgm. of cortisone acetate daily, although it may have arisen on the reduced cortisone dosage just prior to the aldosterone infusion. There is a fairly marked increase in the nitrogen excretion in this patient which seems to be related to the aldosterone infusion. Kekwick and Pawan (4) observed a rise in the nitrogen excretion during aldosterone administration in a patient with Addison's disease. The increase in urinary calcium excretion cannot be assessed from a total balance point of view since the stool calcium was not measured.

Conclusions

A metabolic balance study has been carried out in a totally adrenalectomized patient receiving 600 μgm. of aldosterone intravenously in 24 hr.

Water retention and weight gain without apparent alteration in the sodium and potassium balance was observed. There was no change in the salivary sodium–potassium ratio.

A marked increase in the urinary nitrogen excretion occurred during the aldosterone infusion.

References

1. Beck, J. C., Browne, J. S. L., and MacKenzie, K. R. J. Clin. Endocrinol. and Metabolism, 14(9) : 1006. 1954.
2. Fiske, C. H. and Subbarow, Y. J. Biol. Chem. 66 : 375. 1925.
3. Folin, O. J. Biol. Chem. 82 : 83. 1929.
4. Kekwick, A. and Pawan, G. L. S. Lancet, 2 : 162. 1954.
5. Larsen, C. E. and Greenberg, O. M. J. Biol. Chem. 123 : 199. 1938.
6. Mach, R. S., Fabre, J., Duckert, A., Borth, R., and Ducommon, P. Schweiz. med. Wochschr. 84 : 407. 1954.
7. Peters, J. P. and Van Slyke, D. D. Quantitative clinical chemistry. Vol. 2. Williams & Wilkins Company, Baltimore. 1932. p. 518.
8. Peters, J. P. and Van Slyke, D. D. Quantitative clinical chemistry. Vol. 2. Williams & Wilkins Company, Baltimore. 1932. p. 597.
9. Prunty, F. T. G., McSwiney, R. R., Mills, I. H., and Smith, M. A. Lancet, 2 : 620. 1954.
10. Randolph, T. G. J. Lab. Clin. Med. 34 : 1696. 1949.
11. Roe, J. H. and Kuether, C. A. J. Biol. Chem. 147 : 399. 1943.
12. Simpson, S. A. and Tait, J. F. Ciba Foundation Colloquia Endocrinol. 8 : 1954. In press.
13. Simpson, S. A. and Tait, J. F. Proc. Laurentian Hormone Conf. 10 : 1955. In press.
14. Sodium and Potassium in Foods and Waters. Research Lab., Mead Johnson & Co., Evansville, Ind.
15. Table of Food Values Recommended for Use in Canada by The Nutrition Div. Dept. of Natl. Health and Welfare, Ottawa, Canada.
16. Venning, E. H., Giroud, C. J. P., Dyrenfurth, I., and Beck, J. C. Can. J. Biochem. Physiol. 33 : 605. 1955.
17. Ward, L. E., Polley, H. F., Slocumb, C. H., Hench, P. S., Mason, H. L., Mattox, U. R., and Power, M. H. Proc. Staff Meetings, Mayo Clinic, 29 : 649. 1954.

Notes to Contributors

Manuscripts

(i) *General*

Manuscripts should be typewritten, double spaced, on paper 8½ × 11 in. **The original and one copy are to be submitted.** Tables and captions for the figures should be placed at the end of the manuscript. Every sheet of the manuscript should be numbered.

Style, arrangement, spelling, and abbreviations should conform to the usage of this journal. Names of all simple compounds, rather than their formulas, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed.

Manuscripts and illustrations should be carefully checked before they are submitted. Authors will be charged for unnecessary deviations from the usual format and for changes made in the proof that are considered excessive or unnecessary.

(ii) *Abstract*

An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required, except in Notes.

(iii) *References*

References should be listed **alphabetically by authors' names,** numbered, and typed after the text. The form of the citations should be that used in this journal; in references to papers in periodicals, titles should **not** be given and initial page numbers only are required. All citations should be checked with the original articles, and each one referred to in the text by the key number.

(iv) *Tables*

Tables should be numbered in roman numerals and each table referred to in the text. Titles should always be given but should be brief; column headings should be brief and descriptive matter in the tables confined to a minimum. Vertical rules should be used only when they are essential. Numerous small tables should be avoided.

Illustrations

(i) *General*

All figures (including each figure of the plates) should be numbered consecutively from 1 up, in arabic numerals, and each figure should be referred to in the text. The author's name, title of the paper, and figure number should be written in the lower left-hand corner of the sheets on which the illustrations appear. Captions should not be written on the illustrations (see Manuscripts (i)).

(ii) *Line drawings*

Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper ruled in blue only; any co-ordinate lines that are to appear in the reproduction should be ruled in black ink. Paper ruled in green, yellow, or red should not be used unless it is desired to have all the co-ordinate lines show. All lines should be of sufficient thickness to reproduce well. Decimal points, periods, and stippled dots should be solid black circles large enough to be reduced if necessary. Letters and numerals should be neatly made, preferably with a stencil (**do NOT use typewriting**), and be of such size that the smallest lettering will be not less than 1 mm. high when reproduced in a cut 3 in. wide.

Many drawings are made too large; originals should not be more than 2 or 3 times the size of the desired reproduction. In large drawings or groups of drawings the ratio of height to width should conform to that of a journal page but the height should be adjusted to make allowance for the caption.

The original drawings and one set of clear copies (e.g. small photographs) are to be submitted.

(iii) *Photographs*

Prints should be made on glossy paper, with strong contrasts. They should be trimmed so that essential features only are shown and mounted carefully, with rubber cement, on white cardboard with no space or only a **very** small space (less than 1 mm.) between them. In mounting, full use of the space available should be made (to reduce the number of cuts required) and the ratio of height to width should correspond to that of a journal page (4⅛ × 7⅛ in.); however, allowance must be made for the captions. Photographs or groups of photographs should not be more than 2 or 3 times the size of the desired reproduction.

Photographs are to be submitted in duplicate; if they are to be reproduced in groups one set should be mounted, the duplicate set unmounted.

Reprints

A total of 50 reprints of each paper, without covers, are supplied free. Additional reprints, with or without covers, may be purchased.

Charges for reprints are based on the number of printed pages, which may be calculated approximately by multiplying by 0.6 the number of manuscript pages (double-spaced typewritten sheets, 8½ × 11 in.) and including the space occupied by illustrations. An additional charge is made for illustrations that appear as coated inserts. The cost per page is given on the reprint requisition which accompanies the galley.

Any reprints required in addition to those requested on the author's reprint requisition form must be ordered officially **as soon as the paper has been accepted for publication.**

Contents